

Cdi1, a Human G1 and S Phase Protein Phosphatase That Associates with Cdk2

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Summary

We used the interaction trap, a yeast genetic selection for interacting proteins, to isolate human cyclin-dependent kinase interactor 1 (Cdi1). In yeast, Cdi1 interacts with cyclin-dependent kinases, including human Cdc2, Cdk2, and Cdk3, but not with Cdk4. In HeLa cells, Cdi1 is expressed at the G1 to S transition, and the protein forms stable complexes with Cdk2. Cdi1 bears weak sequence similarity to known tyrosine and dual specificity phosphatases. In vitro, Cdi1 removes phosphate from tyrosine residues in model substrates, but a mutant protein that bears a lesion in the putative active site cysteine does not. Overexpression of wild-type Cdi1 delays progression through the cell cycle in yeast and HeLa cells; delay is dependent on Cdi1 phosphatase activity. These experiments identify Cdi1 as a novel type of protein phosphatase that forms complexes with cyclin-dependent kinases.

Introduction

In eukaryotes, the cell cycle proceeds through two major checkpoints, one before the transition from G1 to S, the other before G2 to M. Many events that result in progression through the G2 checkpoint are intracellular, such as the completion of DNA synthesis (Enoch and Nurse, 1991). By contrast, many events that result in passage from G1 into S, such as the binding of positive and negative growth regulatory proteins to receptors in the cell membrane, are extracellular (Pardee, 1989). In yeast, progress through both checkpoints depends on a cyclin-dependent kinase (Cdk), encoded in *Schizosaccharomyces pombe* by *cdc2+* and in *Saccharomyces cerevisiae* by *CDC28* (reviewed by Nasmyth, 1993). Cell cycle progression in mammals requires multiple Cdks. Cdc2 (Lee and Nurse, 1987) is required for the G2 to M transition (Riabowol et al., 1989). The G1 to S transition requires another Cdk, Cdk2 (reviewed by Pagano et al., 1993; Tsai et al., 1993). Recently, more Cdks have been isolated, including Cdk3, Cdk4, and Cdk5. Although the function of these proteins is not known, it is widely thought that they may also regulate progression

through particular parts of the cell cycle (Meyerson et al., 1992).

As the name suggests, Cdk activity depends on association with cyclins, regulatory molecules synthesized at different times during the cell cycle that complex with Cdks. Availability of cyclins often regulates Cdk activity; in *S. cerevisiae*, the rate-limiting step for the G1/S transition is thought to be the accumulation of G1-specific cyclins, including the products of the *CLN1*, *CLN2*, *CLN3*, *HSC26*, and *CLB5* genes (reviewed by Nasmyth, 1993). Mammalian cells also contain a number of cyclins during G1 to S (cyclins D1, D2, D3, and E; reviewed by Sherr, 1993), and it is likely that changes in their level result in changes in the activity of Cdks present at that time. For example, accumulation of cyclin E and the formation of active cyclin E-Cdk2 complexes are necessary for cells to pass from G1 to S (Tsai et al., 1993; Ohtsubo and Roberts, 1993).

Cdk activity is also regulated by protein modification. In *S. pombe*, during G2, Cdc2 complexed with cyclin B is phosphorylated by the Wee1 and Mik1 tyrosine kinases on Tyr-15 at the ATP-binding site (reviewed by Nillan and Russell, 1992). The phosphorylated complex cannot hydrolyze ATP until it is dephosphorylated by the Cdc25 tyrosine phosphatase (see Moreno et al., 1989); dephosphorylation occurs after DNA synthesis is complete and allows entry into mitosis. A similar mechanism apparently operates in higher cells (Dunphy and Kumagai, 1991; Gautier et al., 1991; Parker and Piwnicka-Worms, 1992). Cdc2 is also modified on at least one other residue, Thr-160, by Cdc2-activating kinase (see Solomon et al., 1993; Poon et al., 1993; Fesquet et al., 1993). Thr-160 phosphorylation is required for binding of cyclins to Cdks and for full Cdk activity (Ducommun et al., 1991; Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993). Although the relevance of phosphorylation to events at G1 to S is not clear, Cdk2, which is required for the G1 to S transition, is modified on the equivalent residues (Gu et al., 1992). The cyclins complexed with Cdks also show intricate patterns of phosphorylation, although the significance of these modifications is not known (see, for example, Hall et al., 1993; Koff et al., 1992).

These regulatory proteins are modulated by other regulators. For example, in *S. pombe* and probably in mammalian cells, at G2/M the Wee1 tyrosine kinase is negatively regulated by the Nim1 product, perhaps in response to signals that DNA replication is complete (see Coleman et al., 1993, and references therein). In *S. cerevisiae*, at G1/S, the activity of G1 cyclins is negatively regulated in response to a proliferation-inhibiting stimulus, α factor. In this case, Fus3 (Elion et al., 1990) phosphorylates Far1 (Chang and Herskowitz, 1990), which in turn binds to Cln1-Cdc28 and Cln2-Cdc28 complexes and inhibits their activity (reviewed by Nasmyth, 1993). Fus3 also inhibits *CLN3* by an unknown mechanism (Elion et al., 1990). In mammalian cells, the upstream regulatory proteins operative at G1/S and G2/M are currently not well understood. The understanding of mammalian Cdk regulators

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and their upstream regulators is important, however, because these proteins will likely help modulate Cdk activity by coupling it to signals inside and outside the cell. Modulation of Cdk activity during G1 is of particular interest because it is during this period that cells typically decide whether to commit to a new round of passage through the cell cycle, to withdraw temporarily from the cycle and enter a nondividing resting state, or to withdraw permanently from the cell cycle and terminally differentiate (Pardee, 1989). Failures in the mechanisms that govern these G1 decisions may underlie some kinds of aberrant differentiation and cancer.

To understand further mammalian G1 decisions, we have sought to isolate proteins involved in the process. To this end we developed a selection (the interaction trap) for genes encoding proteins that bind to known proteins, a selection that relies on work by Fields and Song (1989). We used the trap to isolate a protein, Cdk interactor 1 (Cdi1), that binds human Cdk2. Cdi1 has weak sequence similarity to known tyrosine and dual specificity phosphatases, but is different enough to suggest that it belongs in a novel sequence class. Sequence similarity is most pronounced in an 11 residue region that spans the putative active site. In vitro, Cdi1 dephosphorylates tyrosines in model substrates. Cdi1 phosphatase activity is inhibited by chemical inhibitors and abolished by mutation of a critical cysteine in the active site. When overexpressed, Cdi1 retards cell cycle progression in yeast and mammalian cells. Cell cycle retardation depends on phosphatase activity. *CDI1* mRNA is maximally expressed during G1 and G1/S, and the protein forms stable complexes with a Cdk, Cdk2, operative at this time. Cdi1 does not increase the kinase activity of tyrosine-phosphorylated cyclin-Cdk2 complexes. These experiments are consistent with the idea that the Cdi1 phosphatase might affect cell cycle progression either by controlling phosphorylation of Cdk2-associated proteins or by acting together with Cdk2 complexes on common substrates.

Results

Interaction Trap

To isolate Cdi1, we developed a general transcription-based selection for protein-protein interactions. This interaction trap is an outgrowth of our attempts to use the modular nature of transcription activators (Brent and Ptashne, 1985; Ma and Ptashne, 1988; Triezenberg et al., 1988) to detect protein-protein interactions in yeast. It incorporates a seminal suggestion by Fields and Song (1989) (see Discussion), who, together with other groups, have also developed effective methods for transcription-based interaction cloning (Chien et al., 1991; Dalton and Treisman, 1992; Durfee et al., 1993; Vojtek et al., 1993). Development of the trap, shown in Figure 1a, required careful attention to three classes of components: a fusion protein that contains a LexA DNA-binding domain and that is known to be transcriptionally inert (the "bait"); reporter genes that have no basal transcription and that are bound by the bait; and the proteins encoded by an expression library, which are expressed as chimeras and whose amino termini con-

tain an activation domain and other useful moieties (the "prey"). Relevant design criteria are detailed in Experimental Procedures.

Isolation of Cdi1

We isolated Cdi1 as described (see Experimental Procedures). We rescued library plasmids from cells in which both reporter genes showed galactose-dependent transcription, assigned the plasmids to three different classes by restriction mapping, identified plasmids from each class that contained the longest cDNA inserts, and verified by Western analysis with anti-epitope antiserum that the plasmids directed the synthesis of fusion proteins (data not shown). Detailed restriction mapping and partial DNA

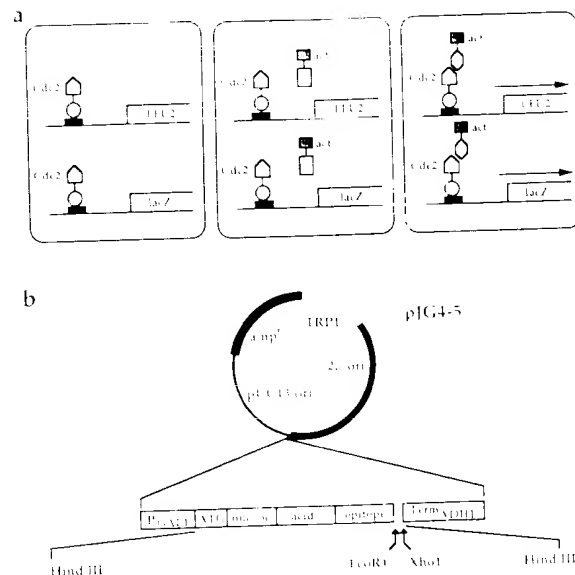
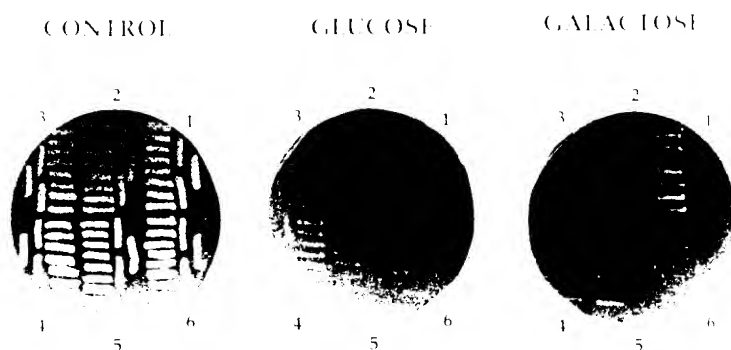


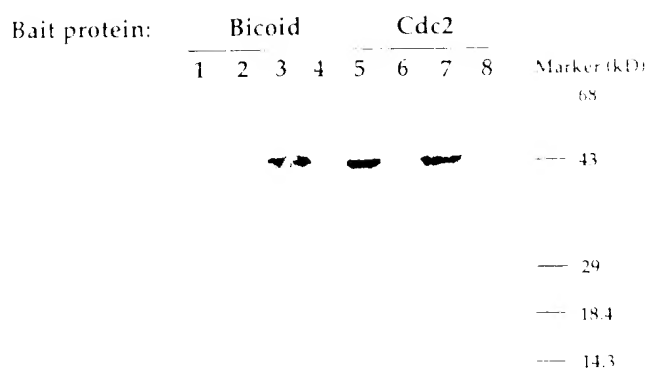
Figure 1. Interaction Trap

(a) (Left) EGY48-p1840 contains two reporter genes, *LexAop-LEU2* and *LexAop-lacZ*, and a transcriptionally inert *LexA-Cdc2* bait. Synthesis of library proteins is induced by growing the yeast in galactose medium. (Middle) The library protein does not interact with the transcriptionally inert bait; the reporter genes are not transcribed; the cell does not form a colony on Leu⁻ medium, and it contains no β -galactosidase activity (act⁻). (Right) The library protein interacts with the bait, and both reporters are active; the cell forms a colony on Leu⁻ medium, cells in the colony have β -galactosidase and are blue on X-Gal medium. (b) The pJG4-5 library plasmid and the invariant 107 amino acid moiety it encodes. This moiety carries amino to carboxyl termini, an ATG, an SV40 nuclear localization sequence (PPKKK RKVA, Kalderon et al., 1984), the B42 transcription activation domain (Ma and Ptashne, 1987), and the HA1 epitope tag (YPYDVPDYA, Green et al., 1982). pJG4-5 directs the synthesis of proteins under the control of a derivative of the *GAL1* promoter. It carries a 2 μ replicator and a *TRP1*⁺ selectable marker. cDNA is inserted on EcoRI-XhoI fragments. Downstream of the XhoI site, pJG4-5 contains the *ADHI* transcription terminator.

a



b



body (Ausubel et al., 1987–1993). Lane 1, galactose, LexA–Bicoid, immunoprecipitation; lane 2, glucose, LexA–Bicoid, immunoprecipitation; lane 3, galactose, LexA–Bicoid, extract; lane 4, glucose, LexA–Bicoid, extract; lane 5, galactose, LexA–Cdc2, immunoprecipitation; lane 6, glucose, LexA–Cdc2, immunoprecipitation; lane 7, galactose, LexA–Cdc2, extract; lane 8, glucose, LexA–Cdc2, extract.

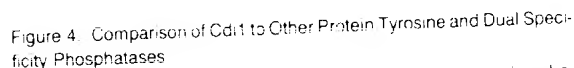
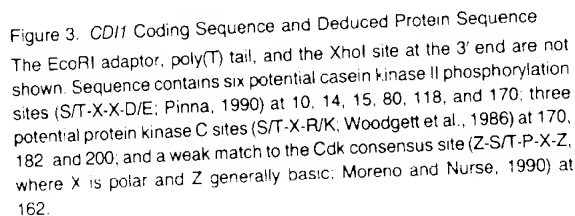
sequencing showed that two of the recovered cDNA classes were previously identified genes *CKS1hs* and *CKS2hs*, the human homologs of the *S. pombe* *suc1⁺* product (Richardson et al., 1990). Sequence of the third cDNA class showed it to be a previously unidentified gene. We called this gene *CDI1* and its protein product Cdi1. We expressed activation-tagged Cdi1 in a panel of EGY48-derived strains containing different baits to test the reproducibility and specificity of its interaction (Figure 2a). As judged by the *LEU2* and *lacZ* transcription phenotypes, Cdi1 interacted specifically with LexA–Cdc2 and did not interact with LexA–c-Myc–C-term, LexA–Max, LexA–Bicoid, LexA–Cln3, or LexA–Fus3 (Figure 2a). Cdi1 did, however, interact with other Cdk's (see below). Specificity of the Cdi1–Cdc2 interaction was confirmed by physical criteria: in vitro, anti-LexA antiserum precipitated epitope-containing proteins from yeast extracts that contained LexA–Cdc2 and Cdi1, but not from extracts that contained LexA–Bicoid and Cdi1 (Figure 2b, lane 5); the mobility of the precipitated proteins was identical to that of immunoreactive Cdi1 bands in extracts of yeast that contained tagged Cdi1 but were not precipitated (Figure 2b, lanes 3 and 7).

Figure 2. Cdi1 Specifically Interacts with Cdc2

(a) In interaction assays. We introduced a library plasmid that expressed tagged Cdi1 into EGY48–p1840 derivatives that contained different baits. We streaked eight individual transformants of the Cdi1-transformed bait strain (horizontal streaks) or the same bait strain plus the library vector as a control (adjacent vertical streaks) onto a master plate, and we replica plated it onto each of three plates: control, Ura⁺ Trp⁺ His⁺ glucose, on which all strains grow; glucose, Ura⁺ Trp⁺ His⁺ Leu[–] glucose, which selects for the transcription of the *LexAop–LEU2* reporter and galactose, Ura⁺ Trp⁺ His⁺ Leu[–] galactose, which selects for transcription of the *LexAop–LEU2* reporter and on which Cdi1 is expressed. Baits were LexA–Cdc2 (1), LexA–Bicoid (2), LexA–Max (3), LexA–Cln3 (4), LexA–Fus3 (5), and LexA–c-Myc–C-term (6). Note that on glucose the Cln3 bait weakly activated the *LexAop–LEU2* reporter, but that on galactose the inferiority of the carbon source and diminished bait expression from the *ADHI* promoter (Brant and Prashne, 1984) eliminated this background. (b) In immunoprecipitations. We grew 100 ml cultures of EGY48 that contained a Cdi1-expressing library plasmid and either LexA–Cdc2 or LexA–Bicoid in galactose or glucose medium and then prepared cell extracts as described (Wittenberg and Reed, 1988). Extract (5 μ l) was taken as a control, and rabbit anti-LexA antiserum (15 μ l) was used to immunoprecipitate LexA fusion proteins as described (Wittenberg and Reed, 1988). Pellets were dissolved in Laemmli sample buffer, run on a 12.5% SDS protein gel, and blotted onto nitrocellulose. Tagged Cdi1 was identified by Western analysis of the blotted proteins with the 12CA5 monoclonal anti-hemagglutinin anti-

The Cdi1 Protein

CDI1 was isolated from 12 different library plasmids that contained cDNAs of four different lengths. Sequence analysis revealed that all *CDI1* cDNA inserts contained an open reading frame, and inspection of the sequence of the longest cDNAs revealed an ATG with a perfect match to the consensus translation initiation sequence (PuCC/GATGG, Kozak, 1986) (Figure 3a). Careful analysis of the size of *CDI1* mRNA in HeLa cells (data not shown) revealed that this ATG occurs between 15 and 45 nt from the 5' end of the *CDI1* message, suggesting that the longest cDNAs spanned the entire open reading frame, which spans 212 codons. Since isolated *CDI1* cDNAs by definition encoded proteins that interacted with Cdc2, the sizes of *CDI1* cDNA inserts from this hunt necessarily localized the portion of the protein sufficient for interaction with Cdc2 to the carboxy-terminal ~170 amino acids of Cdi1. The Cdi1 sequence (Figure 3a) revealed previously identified protein motifs. First, at the amino terminus, 19 out of 35 amino acids are either proline, glutamic acid, serine, or threonine. Proteins that contain these stretches, called PEST sequences, are degraded rapidly (Rogers et al., 1986), and this stretch of Cdi1 is more enriched in these amino



(a) Comparison of Cdd1 and human placental protein tyrosine phosphatase 1B (PTP1b; Charbonneau et al., 1989). Sequences were aligned by the GAP algorithm (Pearson and Lipman, 1988). Vertical lines indicate identity; colons and periods indicate conserved changes. Highly conserved residues found in all phosphatases are indicated by asterisks (Freeman et al., 1992).

(b) Comparison of the active site region of Cdi1 to the active site region of other tyrosine phosphatases as aligned by Gautier et al. (1991). The top block includes human T cell protein tyrosine phosphatase (HsTCPTP; Cool et al., 1989), human protein tyrosine phosphatase 1B (PTP1B; human leukocyte common antigen-related protein domain 1 (HsLAR D1; Streuli et al., 1988), and the *S. pombe* (SpPYP1; Ottilie et al., 1991), *S. cerevisiae* (ScPTP1; Guan et al., 1991b), and vaccinia virus (VH1) (Guan et al., 1991a) tyrosine and dual specificity phosphatases. The bottom block of Cdc25 proteins are from human (HsCdc25; Galaktionov and Beach, 1991; Sadhu et al., 1990), *Drosophila* (Dmstr, Edgar and O'Farrell, 1989), *S. cerevisiae* (ScMIH1; Russell et al., 1989), and *S. pombe* (Spcdc25, Russell and Nurse 1986). In the top block, the highly conserved active site histidine, cysteine, glycine, and arginine residues are shown in bold; in the Cdc25 proteins, the conserved glutamic acid and the serine that replaces glycine in the active site are also in bold.

Protein Phosphatase Activity of Cdi1
We tested the ability of Cdi1 to dephosphorylate phosphotyrosine-containing substrates. We altered the Cdi1 codon corresponding to the catalytic cysteine (Pot and Dixon, 1992) in the tyrosine phosphatase active site consensus

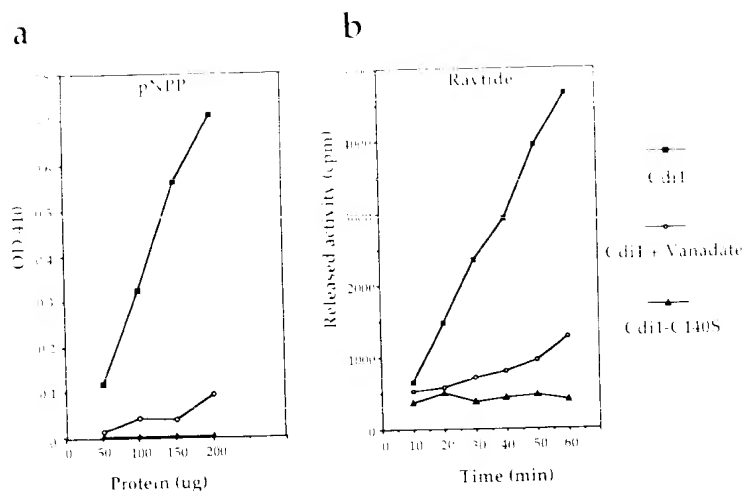


Figure 5. Cdi1 Phosphatase Activity

(a) Hydrolysis of pNPP by GST-Cdi1 proteins. We incubated 20 mM pNPP with indicated amount of proteins as described in Experimental Procedures. Squares, Cdi1; circles, Cdi1 with 0.5 μM Na₂VO₄; triangles, Cdi1-C140S.

(b) Time course of ³²P-labeled Raytide dephosphorylation by GST-Cdi1. We incubated 10 cpm of Raytide with 10 μg of indicated proteins for the indicated times at 30°C as described in Experimental Procedures. Squares, Cdi1; circles, Cdi1 with 0.5 μM Na₂VO₄; triangles, Cdi1-C140S.

(C140) to one encoding serine (see Experimental Procedures). We produced Cdi1 and Cdi1-C140S in *Escherichia coli* as glutathione S-transferase (GST) fusions and purified them to ~90%–95% homogeneity, as judged on Coomassie-stained SDS protein gels (data not shown). We used the purified proteins in phosphatase assays with model substrates. First, we utilized the chromogenic substrate para-nitrophenyl phosphate (pNPP; Sigma). Wild-type Cdi1 efficiently hydrolyzed pNPP. Hydrolysis increased linearly as a function of Cdi1 concentration (Figure 5a). Cdi1-C140S did not catalyze detectable pNPP hydrolysis (Figure 5a). Sodium vanadate, an inhibitor of the known phosphotyrosine-specific phosphatases (Charbonneau and Tonks, 1992), effectively blocked hydrolysis by wild-type Cdi1 (Figure 5a). In 2 hr incubations, 1 molecule of GST-Cdi1 catalyzed hydrolysis of ~140 pNPP molecules (1.2 mol/min). On a molar basis, Cdi1 was 8–12 times more active than similarly purified full-length human Cdc25C (data not shown).

We then tested the ability of Cdi1 to dephosphorylate a peptide substrate. For this purpose we labeled a peptide (Raytide; Oncogene Sciences, Incorporated, Manhasset, New York) with *c*-Src tyrosine kinase and [γ -³²P]ATP. We incubated labeled peptide in the presence or absence of Cdi1 and measured released ³²PO₄ (see Experimental Procedures). Results with this substrate were similar to those with pNPP. Wild-type Cdi1 efficiently removed phosphate from the peptide. Sodium vanadate inhibited dephosphorylation. Cdi1-C140S displayed no activity (Figure 5b). We also tested whether Cdi1 functioned as a serine/threonine phosphatase by determining whether it could remove phosphates from ³²P-labeled H1 histone phosphorylated by Cdc2; in our hands, it did not (data not shown). The above results demonstrate that Cdi1 is a protein tyrosine phosphatase and suggest that it is not active against serines and threonines.

Cdi1 Expression in HeLa Cells

Because it interacts with Cdks, we explored the possibility that the Cdi1 phosphatase might affect cell cycle progres-

sion. We first examined Cdi1 expression during the cell cycle. We synchronized exponentially growing adherent HeLa cells by two cycles of treatment with high concentrations of thymidine to arrest them in late G1, followed by release from this block by exposure to normal concentrations of thymidine, measured their progression through the cell cycle by fluorescence-activated cell sorting (Figure 6a), and isolated RNA at different times. These cells entered S phase more slowly after release from block than reported for suspension HeLa cells (Lew et al., 1991). As is shown in Figure 6b, expression of *Cdi1* mRNA peaks during late G1 and early S. Accumulation of Cdi1 protein follows the same pattern, as judged by Western blots with anti-Cdi1 antiserum (data not shown). In these experiments, two reference mRNAs showed the expected time of expression: cyclin E mRNA was expressed late in G1 and disappeared by late S phase (Lew et al., 1991), while cyclin B1 mRNA was expressed later, appearing in S phase and disappearing in M (Pines and Hunter, 1989).

This timing of expression suggested that Cdi1 phosphatase might function during late G1, during the G1 to S transition, or during early S. To test this idea, we stably transfected HeLa cells with a plasmid that expressed Cdi1 from the Moloney murine leukemia virus long terminal repeat and includes the *neo* resistance gene. Compared with the vector, the Cdi1-expressing construction did not yield a reduced number of G418-resistant transformants. However, fluorescence-activated cell sorting analysis of DNA in Cdi1-expressing transformants showed a significant and reproducible increase in the proportion of cells in G1 or early S relative to control populations (Figure 6c), suggesting that Cdi1 overexpression may retard passage through these phases of the HeLa cell cycle.

Cdi1 Expression in Yeast Cells

Cdi1 also inhibited cell cycle progression in yeast. Cultures of *S. cerevisiae* that expressed Cdi1 increased their cell number and optical density more slowly than control populations (Figure 7a; data not shown). Microscopic examination of these cells showed that, compared with controls,

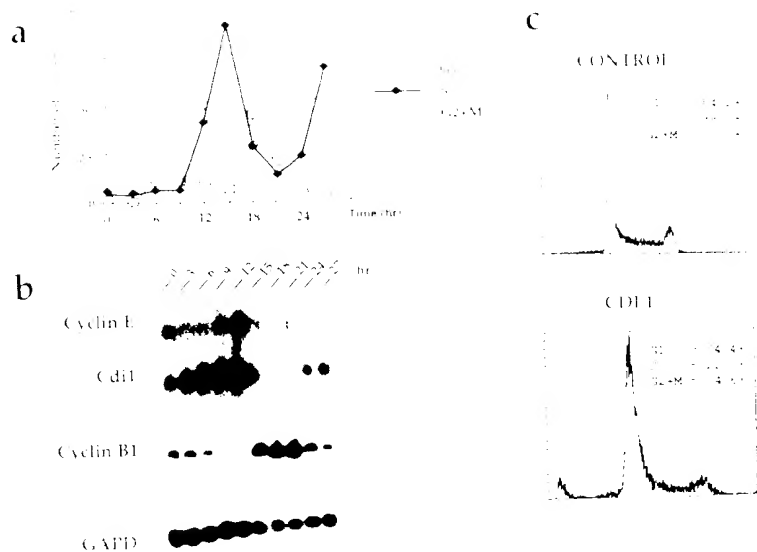


Figure 6. *Cdi1* Expression in HeLa Cells.

(a and b) Timing. We synchronized adherent HeLa cells by a double thymidine block (Lew et al., 1991) and collected aliquots every 3 hr after release. (a) Fluorescence-activated cell sorting analysis of DNA content, which shows that cells reentered the cell cycle 9 hr after release. (b) Northern blot analysis of RNAs. RNA from each aliquot was run on a formaldehyde-agarose gel and blotted onto nylon as described (Ausubel et al., 1987-1993). The blot was probed with random-primed DNA probes from *Cdi1*, human cyclin E, human cyclin B1, and human glyceraldehyde-phosphate-dehydrogenase (GAPD), a normalization control. Lane labels show times in hours after release.

(c) Effect. HeLa cells were transfected either with pBNCdi1 (see Experimental Procedures), which directed the synthesis of Cdi1, or with vector alone and were analyzed as described in Experimental Procedures. The G1 midpoint is defined as the mode of the distribution of each graph; the modes on the two panels are of different heights (272 counts for cells transformed with the vector, 101 counts for cells that contain Cdi1); the broadened peak in the Cdi1 cells reflects the increased proportion of the population containing approximately $1 \times$ DNA content. Results of a typical run are shown.

Cdi1-expressing cells were larger and, when stained with DAPI, that the nuclei of some of the largest cells were not condensed (data not shown). Less than 10% of the cells in Cdi1-expressing populations showed buds, as opposed to 40% of the cells in the control population, suggesting that more of the cells in the Cdi1-expressing population were in G1 and that Cdi1 overexpression causes a retardation in passage through the cell cycle similar to its effect in mammalian cells (Figure 7b).

We examined two models for Cdi1 inhibition of cell cycle

progression. First, because Cdi1 interacts with Cdc28 (see below), its growth retardation phenotype might be due to sequestration of yeast Cdc28 into protein complexes that were not competent to cause the cell to traverse G1 and early S. If so, then the phenotype should be reversible by co-overproducing the human Cdc2 against which Cdi1 was selected. Second, because Cdi1 is a protein phosphatase, its inhibitory effect might be dependent on its phosphatase activity. To test these ideas, we expressed wild-type Cdi1 and the Cdi1-C140S mutant in cells that

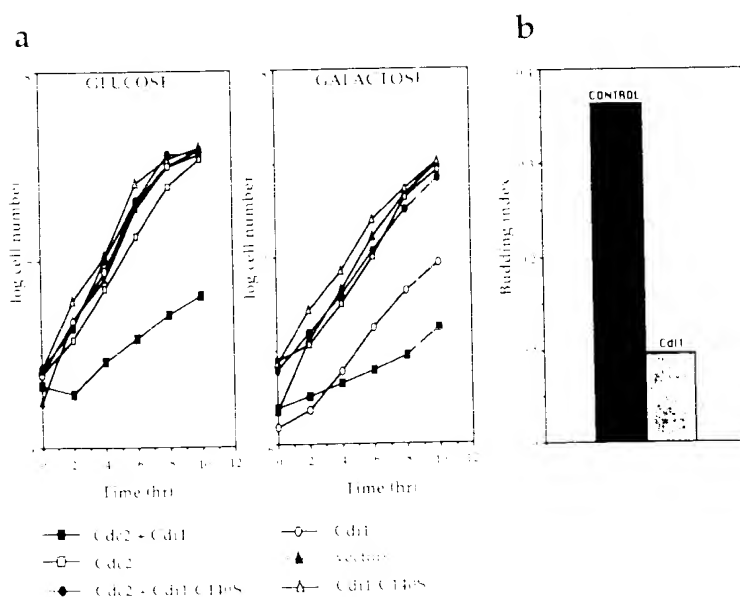


Figure 7. Effect of Cdi1 on Yeast Cell Growth.

(a) Growth rates of cells that expressed Cdi1. *S. cerevisiae* W303 that carried the indicated combinations of Cdi1 and/or Cdc2 expression vectors were grown in the indicated media and growth monitored by OD₆₀₀ as described in Experimental Procedures. Note that the Cdi1 and Cdc2 expression plasmids together caused some growth inhibition even in glucose medium, which we attribute to leaky expression from the *GAL1* promoter derivative on the pJG4-1 expression plasmid (J. G., unpublished data). Closed triangles, expression vectors only; open squares, Cdc2; open circles, wild-type Cdi1; open triangles, Cdi1-C140S; closed squares, Cdc2 and wild-type Cdi1; closed circles, Cdc2 and Cdi1-C140S.

(b) Budding index. Cells that expressed Cdi1 or contained the expression vector were grown for 6 hr in galactose as described above. Cells (400 from each population) were examined by phase-contrast microscopy, and the budding index was calculated as the percentage of budded cells in each population.

contained Cdc28, with and without overexpressed native human Cdk2.

Figure 7a shows that coexpression of Cdc2 did not rescue Cdi1-dependent growth inhibition. The Cdi1-C140S mutant did not cause growth inhibition, whether it was expressed alone or together with Cdc2 (Figure 7a). Unexpectedly, coexpression of Cdc2 with wild-type Cdi1 increased the severity of the Cdi1-dependent growth inhibition by about 2-fold (Figure 7a). These experiments suggest three conclusions. First, because expression of excess Cdc2 does not rescue Cdi1 growth retardation, they suggest that this growth inhibition cannot be explained by simple sequestration of Cdc28 into nonfunctional complexes. Second, because Cdi1-dependent growth inhibition is strictly dependent on the phosphatase activity of Cdi1, these results suggest that the inhibition is due to dephosphorylation of Cdi1 substrate(s). Finally, because Cdi1-dependent growth inhibition is heightened by coexpression of Cdc2, these results suggest a functional genetic interaction between Cdi1 and Cdc2 in addition to the physical interaction.

Cdi1 Activity In Vitro

We considered two explanations for the genetic interaction between Cdi1 and Cdk2 suggested by the yeast coexpression experiments. First, just as Cdc2 is reported to phosphorylate Cdc25 and increase its phosphatase activity (Hoffman et al., 1993), we thought that Cdc2 might phosphorylate Cdi1 and increase its activity. We thus tested whether known Cdk2s could phosphorylate Cdi1 in vitro. We found that neither Cdc2 nor Cdk2 complexes isolated from HeLa cells nor cyclin B1-Cdk2 complexes reconstituted from pure proteins expressed in Sf9 cells (a gift of B. Gabrielli and H. Piwnicka-Worms) could phosphorylate GST-Cdi1, although these complexes phosphorylated histone H1 (data not shown). Second, we asked whether Cdi1 activated Cdk2s. For this purpose we used a cyclin B1-Cdk2 complex phosphorylated in vitro on Tyr-15 by the human Wee1 tyrosine kinase (a gift of B. Gabrielli and H. Piwnicka-Worms). Treatment of this complex with GST-Cdi1 did not increase its histone H1 kinase activity, while treatment with GST-Cdc25C did (data not shown). This suggested that Cdi1 is unable to remove the inhibitory phosphate from Tyr-15 and to activate the kinase. Thus, although Cdi1 phosphatase and Cdk2s interact, these results suggest that neither protein is a substrate for the other.

Cdi1 Interaction with Cdk2s in Yeast

Since Cdi1 is normally expressed during G1 and its overexpression retards the G1 to S transition, it seemed possible that it might normally form complexes with Cdk2s other than Cdc2. To determine which Cdk2s might be Cdi1 partners, we used the interaction trap to measure the ability of Cdi1 to interact with a panel of different bait proteins. This panel included LexA derivatives of Cdc2 proteins from yeast (Lorincz and Reed, 1984), humans (Lee and Nurse, 1987), and flies (Jimenez et al., 1990; Lehner and O'Farrell, 1990). LexA derivatives of human Cdk2

(Elledge and Spotswood, 1991; Tsai et al., 1991) and Cdk3 (Meyerson et al., 1992), and LexA derivatives of the less closely related human Cdk4 (Hanks, 1987) and yeast Fus3 (Elion et al., 1990).

Table 1 shows that activation-tagged Cdi1 stimulated transcription from these baits to different levels: Cdi1 activated strongly in strains that contained the human Cdc2 bait against which it was selected, less strongly in strains that contained *S. cerevisiae* Cdc28, human Cdk2, or human Cdk3 baits, and only weakly in strains that contained DmCdc2, one of the two *Drosophila* Cdc2 homologs. In strains that contained human Cdk4, DmCdc2c, or Fus3 baits, Cdi1 did not activate at all (Table 1). All kinases with which Cdi1 interacted contained the so-called PSTAIRE motif, a stretch of amino acids that includes the Cdk catalytic site (Meyerson et al., 1992). Cdi1 did not interact with Cdk4, which has a similar sequence in this region (PISTVRE; Meyerson et al., 1992), or with Fus3, which has no apparent similarity to this region. Cdi1 did not interact with DmCdc2c, which contains a PSTAIRE motif but which differs substantially at other residues. This experiment suggested that the partners of Cdi1 in mammalian cells may be other PSTAIRE-containing Cdk2s present when Cdi1 is, such as Cdk2.

Association of Cdi1 with Cdk2 in HeLa Cells

To explore the Cdi1-Cdk2 interaction, we used anti-Cdi1 antiserum (tested as described in Experimental Procedures) and other antisera that specifically recognized Cdc2 and Cdk2 (gifts of L.-H. Tsai and E. Harlow) to precipitate complexes containing these proteins from ³⁵S-labeled HeLa cell extracts. Precipitated proteins were separated on SDS gels (Figure 8a). Cdi1 antiserum brought down major protein bands with apparent molecular masses of 21 kd and 33 kd (Figure 8a, lane 3). These bands were absent from precipitates that used Cdi1 preimmune serum (Figure 8a, lane 2) and nonspecific rabbit serum (lane 6). The 21 kd band corresponded to Cdi1; its mobility was the same as that of in vitro translated Cdi1 and of immunoprecipitated in vitro translated Cdi1 (see Experimental Procedures). The 33 kd band had a similar mobility to one of the two major bands immunoprecipitated from HeLa

Table 1. Differential Interaction of Cdi1 with Cdc2 Family Members

Bait	Species	β -Galactosidase Activity
LexA-Cdc2	Human	1240
LexA-Cdk2	Human	470
LexA-Cdk3	Human	460
LexA-Cdk4	Human	<2
LexA-Cdc28	<i>S. cerevisiae</i>	390
LexA-Cdc2	<i>Drosophila</i>	40
LexA-Cdc2c	<i>Drosophila</i>	<2
LexA-Fus3	<i>S. cerevisiae</i>	<2

EGY48-JK103 contained tagged Cdi1 and plasmids that expressed different LexA-Cdk baits: human Cdc2, Cdk2, Cdk3, and Cdk4; *S. cerevisiae* Cdc28 and Fus3; and *Drosophila* Cdc2 and Cdc2c. We measured β -galactosidase activity of five isolates of each strain grown in 2% galactose; variation among individual transformants was less than 20%.

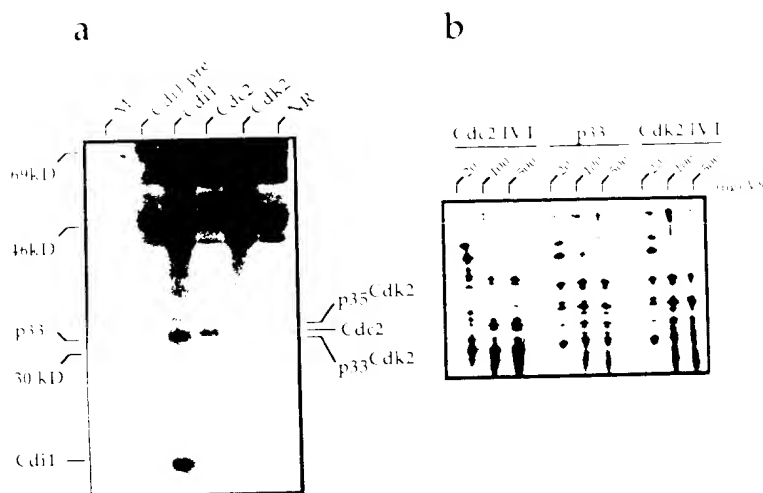


Figure 8 Cdi1-Associated Proteins in ³⁵S-Labeled HeLa Cell Extracts

Proteins were immunoprecipitated from HeLa cell extracts and analyzed as described in Experimental Procedures.

(a) Molecular mass markers (M) (lane 1), pre-immune serum (lane 2), and anti-Cdi1 (lane 3), anti-Cdc2 (lane 4), anti-Cdk2 (lane 5), and nonspecific rabbit (NR) (lane 6) antisera.

(b) Partial proteolysis of proteins with indicated amounts of *S. aureus* V8 protease was as described (see Experimental Procedures). Lanes 1–3, in vitro translated (IVT) Cdc2; lanes 4–6, p33 band from anti-Cdi1 immunoprecipitations; lanes 7–9, in vitro translated (IVT) Cdk2.

lysates with the anti-Cdk2 antisera (Figure 8a, lane 5). The mobility of these two bands matched published reports in which the 33 kD band corresponds to Thr-160-phosphorylated Cdk2 (p33^{Cdk2}), and the 35 kD band corresponds to Cdk2 that is not phosphorylated at this residue (p35^{Cdk2}; Gu et al., 1992). These results suggested that Cdi1 might form complexes with p33, the active form of Cdk2. Comparison of the bands in the Cdi1 precipitation to those in the anti-Cdc2 precipitation (Figure 8a, lane 4) suggested that in HeLa cells Cdi1 does not associate with Cdc2. Cdk2 antiserum did not precipitate detectable amounts of the 21 kD Cdi1 band (Figure 8a, lane 5), suggesting that Cdi1 might make essential contacts with the stretch of the Cdk2 carboxyl terminus to which the antiserum was raised. Cdi1 antiserum brought down other proteins of higher molecular mass. Three of these bands, of apparent molecular masses of 45–63 kD, were also precipitated with anti-Cdk2 antiserum. From their molecular masses, these bands may be the Cdk2-associated cyclins A (63 kD), B1 (55–56 kD), and E (45–50 kD) (Pines and Hunter, 1989; Draetta and Beach, 1988; Tsai et al., 1993).

To confirm the tentative identification of the Cdi1-associated 33 kD band as p33^{Cdk2}, we subjected the ³⁵S-labeled 33 kD protein band to partial proteolysis with V8 and analyzed the products on SDS gels (Harlow and Lane, 1988). We compared the pattern of bands with that given by similarly treated human Cdk2 synthesized by translation in vitro (see Experimental Procedures). The pattern resulting from digestion of the 33 kD band was identical to that obtained from Cdk2, indicating that in HeLa cells one partner of Cdi1 is Cdk2 (Figure 8b, lanes 4–9). This pattern was distinctly different from the pattern resulting from digestion of Cdc2 (Figure 8b, lanes 1–3).

Discussion

Passage of eukaryotic cells through different phases of the cell cycle is dependent on Cdks. Interacting regulatory proteins connect the activity of the Cdks to signals generated inside the cell and to events in the extracellular environment (Sherr, 1993). To understand better cell cycle

regulation in humans, we developed a general yeast transcription genetic selection and used it to isolate proteins that touch Cdks and might modulate their activity or function with them during the cell cycle. This selection, the interaction trap, relies on the fact that eukaryotic transcription activators are often composed of discrete DNA-binding and activation domains and that the activation domain can be brought to the promoter by protein–protein interactions (Brent and Ptashne, 1985; Ma and Ptashne, 1988; Fields and Song, 1989). It is especially dependent on the two-hybrid system proposed by Fields and Song (1989) in that our use of transcription to detect proteins that interact with DNA-bound baits incorporates their suggestion that potential interacting proteins could be detected if they carried activation domains. Several techniques that make use of this suggestion have been developed in a number of labs (Chien et al., 1991; Dalton and Treisman, 1992; Durfee et al., 1993; Vojtek et al., 1993).

As demonstrated here and in Zervos et al. (1993), a number of features make the trap particularly useful. First, the reporters have qualitatively different phenotypes: the *LexAop-LEU2* reporter provides a selection, allowing large numbers of potential interactors to be easily surveyed, while the *LexAop-lacZ* reporter provides a simple secondary test to reject adventitious *LEU*⁺ colonies. Second, the reporters have different promoters, which may eliminate false positive proteins that interact with the promoter instead of the bait. Third, the reporter phenotypes differ in their sensitivity, which affects the affinity of the bait–prey interactions they can detect. The *LexAop-LEU2* reporter responds to interactions with an apparent *K*₀ of about 10^{–6} M, while the different *LexAop-lacZ* reporters respond with different sensitivities; which *lacZ* reporter is used is determined by the investigator. Fourth, the activation region on the library proteins is relatively weak, to avoid restrictions in the spectrum of recovered proteins that might be caused by the strong, semitoxic activation domains of GAL4 and VP16 (Gill and Ptashne, 1988; Triezenberg et al., 1988; Berger et al., 1992). Finally, conditional expression from the library vector allows the *Leu*

and LacZ phenotypes to be unambiguously ascribed to the library protein, diminishing the number of library plasmids that must be excluded by subsequent tests. While useful schemes with some of these attributes have recently been described (e.g., Vojtek et al., 1993), none has the entire set.

Because this interactor hunt used a relatively insensitive *lacZ* reporter, it identified only three proteins, all of which associate strongly with human Cdc2. Two had been previously identified: the CKS1 and CKS2 proteins (Richardson et al., 1990), the human homologs of the *S. pombe* *suc1⁺* product. Cdi1, the protein reported here, contains 212 amino acids, of which the carboxy-terminal 170 are sufficient for interaction with Cdc2. Cdi1 has consensus sites for protein kinase C and casein kinase II. The amino-terminal 35 residues of the protein comprise a PEST sequence (Rogers et al., 1986). Cdi1 possesses weak sequence similarity to the catalytic domain of protein tyrosine and dual specificity phosphatases, including similarity to a stretch of amino acids that defines the active site (Charbonneau and Tonks, 1992). From sequence, Cdi1 is more different from known nonreceptor tyrosine and dual specificity phosphatases than these are to one another, and it apparently lacks the regulatory domain found in all these proteins except the VH1-related phosphatases (Charbonneau and Tonks, 1992). In vitro, Cdi1 possesses tyrosine phosphatase activity on model substrates such as pNPP and tyrosine-phosphorylated peptides. Cdi1 activity is inhibited by chemical inhibitors and by a mutation that eliminates a cysteine in the catalytic site. These facts establish that Cdi1 represents a novel class of human protein phosphatase, which, like *S. cerevisiae* Cdc14 and Cdc25, may function in cell cycle progression (Millar and Russell, 1992; Wan et al., 1992).

In HeLa cells that express Cdi1 under the control of a retroviral promoter, the proportion of cells in the population in G1 or early S is increased, suggesting that Cdi1 overexpression delays progression beyond these stages of the cell cycle. This fact is consistent with the time of its expression, which is strikingly specific for the cell cycle stage: in synchronized populations, *Cdi1* mRNA is highest during G1 and at the G1 to S transition. Expression of Cdi1 also retards cell cycle progression in *S. cerevisiae*; again, Cdi1 increases the proportion of cells in G1, suggesting that expression retards the G1 to S transition. Retardation requires Cdi1 phosphatase activity and is enhanced by overexpression of Cdc2. These results indicate that cell cycle retardation by Cdi1 is not likely due to sequestration of Cdc28 by Cdi1 into inactive complexes and suggest a functional genetic interaction between Cdi1 and Cdk.

As measured by interaction in yeast, Cdi1 interacts with different Cdk with very different affinities. All Cdk with which Cdi1 interacts strongly (Cdc2, Cdk2, and Cdk3) contained a distinctive set of amino acids in the active site, the so-called PSTAIRE motif (Meyerson et al., 1992). To identify those proteins complexed with Cdi1 in human cells, we immunoprecipitated Cdi1-associated proteins from HeLa cells. The apparent molecular mass of one of these (33 kd) suggested that it might be the Thr-160-phosphorylated form of Cdk2. We analyzed this protein by partial V8 proteolysis and confirmed that the protein

was Cdk2. We were unable to demonstrate an association of Cdi1 with other Cdk in HeLa extracts. These experiments demonstrate that Cdi1 associates with Cdk2, a kinase thought to govern the G1 to S transition, and thus further emphasize that Cdi1 may influence this transition.

The most obvious model for Cdi1 function, given its association with Cdk2, is that Cdi1 function might modify Cdk2, dephosphorylating the Tyr-15 equivalent and stimulating kinase activity. However, since we cannot activate tyrosine-phosphorylated Cdk2-containing complexes by treatment with Cdi1, we do not think it removes tyrosines from Cdk2. A second model is that Cdi1 dephosphorylates other Cdk2-associated proteins, such as cyclins, thus indirectly affecting Cdk activity; this idea receives support from the fact that complex patterns of cyclin A, B1, D1, and E phosphorylation exist (see, for example, Hall et al., 1993; Koff et al., 1992). A third model is that Cdi1 functions with other members of Cdk2-containing complexes on common substrates. This idea is consistent with the observed functional interaction between Cdi1 and Cdk in yeast in which coexpression of Cdc2 increases Cdi1-dependent cell cycle retardation. In this view, the proteins complexed with Cdi1 may provide the regulatory domain that Cdi1 apparently lacks. Identification of the Cdi1 substrate(s) will help distinguish among these ideas and in understanding the function of this Cdk2-associated phosphatase during G1 and early S.

Experimental Procedures

Bacteria and Yeast

Manipulation of *E. coli* and DNA was by standard methods (Ausubel et al., 1987–1993). "Sure" *mcrA*Δ (*mrr*, *hsdRMS*, *mcrBC*) *endA*1 *supE*44 *thi*1 *gyrA*96 *relA*1 *lac* *recB* *recJ* *sbcC* *umuC* *Tn5* (*kan*^r) *uvrC*1 *F*[*proAB*, *lacZ*Δ100] *Tn10*(*ter*^r) (Stratagene, Incorporated) and *KC8* *pyrF* *Tn5* *hsdR* *leuB*600 *trpC*9830 *lac*Δ174 *strA* *galK* *hisB*436 (a gift of K. Struhl) were used as hosts. The growth rate experiments used W303 (*MATa* *trp1* *ura3* *his3* *leu2* *can1* *bar1*Δ::*LEU2*) (a gift of M. Whiteway) that contained either pJG4-4Cdi1 (J. G., unpublished data), a plasmid that directs the synthesis of native Cdi1, or a vector control and also contained either pJG14-2 (J. G., unpublished data) or a derivative that directs the synthesis of native human Cdc2 in yeast. These were grown overnight at 30°C in 2% raffinose His⁺ Trp⁺, collected, washed, and diluted to an OD₆₀₀ of 0.1 into fresh medium that contained either 2% glucose or 1% galactose plus 1% raffinose. ODs were taken every 2 hr.

Interaction Trap

Detailed map, sequence, and methodological information is available on request and from the Massachusetts General Hospital (MGH) Molecular Biology Internet Gopher server. Baits were produced constitutively from a 2μ *HIS3⁺* plasmid under the control of the *ADHI* promoter and contained the LexA carboxy-terminal oligomerization region, which contributes to operator occupancy by LexA derivatives, perhaps by precisely aligning LexA amino termini on adjacent operator half sites (Golemis and Brent, 1992). Note that most LexA derivatives enter the nucleus in concentrations sufficient to permit operator binding, even though LexA derivatives are not specifically nuclear localized unless they contain other signals (Golemis and Brent, 1992; Silver et al., 1986). Baits were made in LexA(1-202)+PL (Ruden et al., 1991), a close relative of pMA42, pSH2-1, and pEG202 (Hanes and Brent, 1989; Ma and Ptashne, 1987; E. G. and R. B., unpublished data) as follows: human *Cdc2* (Lee and Nurse, 1987), *Cdk2* (Tsai et al., 1991), and *Cdk3* (Meyerson et al., 1992) and *S. cerevisiae* *CDC28* (Lorincz and Reed, 1984) coding sequences were amplified by polymerase chain reaction from plasmids that contained them with Vent polymerase (New England Biolabs) and were inserted into LexA(1-202)+PL as EcoRI–BamHI fragments. LexA–Cdk4 (Hanks, 1987; Meyerson et

al., 1989) was a gift of R. V. Svanathan. These proteins contained two amino acids (EF) between the last amino acid of LexA and the bait moieties. The *Drosophila* Cdc2 baits (Lehner and Farrell, 1990) were gifts of R. Finley. LexA-Fus3 (Elion et al., 1990) and LexA-Clr3 (Cross, 1988) were made analogously except they were cloned as BamHI fragments and thus contained five amino acids (EFGI) between LexA and the fused moieties. All these fusions contained the second codon to the stop codon of each bait. LexA-c-Myc-C-term (which contains the carboxy-terminal 176 amino acids of human c-Myc), LexA-Max (which contains all of the human Max coding sequence), and LexA-Bicoid (amino acids 2–160), a gift of R. Finley, have been described (Zervos et al., 1993; Golemis and Brent, 1992).

Reporters

The *LexAop-LEU2* construction replaced the yeast chromosomal *LEU2* gene. The other reporter, pRB1840, one of a series of *LexAop-GAL1-lacZ* genes (Brent and Ptashne, 1985; Kamens et al., 1990; S. Hanes, unpublished data), was carried on a 2 μ plasmid. Basal reporter transcription was extremely low, presumably owing both to the removal of the entire upstream activating sequence from both reporters and to the fact that LexA operators introduced into yeast promoters decrease their transcription (Brent and Ptashne, 1984). Reporters were chosen to differ in sensitivity. The *LEU2* reporter contained three copies of the high affinity LexA-binding site found upstream of *E. coli* *colE1* (Ebina et al., 1983; Kamens et al., 1990), which presumably bind a total of six dimers of the bait. In contrast, the *lacZ* gene contained a single lower affinity operator (Brent and Ptashne, 1984) that binds a single dimer of the bait. The operators in the *LEU2* reporter were closer to the transcript start point than they were in the *lacZ* reporter. These differences in the number, affinity, and operator position all contribute to that fact that the *LEU2* reporter is more sensitive than the *lacZ* gene. Construction of the EGY48 selection strain will be detailed elsewhere (E. G., unpublished data).

Expression Vectors and Library

Library proteins were expressed from pJG4-5, a member of a series of expression plasmids designed to be used in the interaction trap and to facilitate analysis of isolated proteins. These plasmids carry the 2 μ replicator and the *TRP1* marker. pJG4-5, whose construction will be described elsewhere, directs the synthesis of fusion proteins. Proteins expressed from this vector possess the following features: galactose-inducible expression so that their synthesis is conditional; an epitope tag to facilitate detection; a nuclear localization signal to maximize intranuclear concentration to increase selection sensitivity; and an activation domain derived from *E. coli* (Ma and Ptashne, 1987), chosen because its activity is not subject to known regulation by yeast proteins and because it is weak enough to avoid toxicity (Gill and Ptashne, 1988; Berger et al., 1992) that might restrict the number or type of interacting proteins recovered. In addition to this plasmid, we used two Cdi1 expression plasmids (J. G., unpublished data). We introduced EcoRI-XhoI Cdi1-containing fragments into pJG4-4 to make pJG4-4Cdi1; Cdi1 was expressed from this plasmid as a native, untagged protein under the control of the *GAL1* promoter. We also introduced EcoRI-XhoI fragments that contained Cdi1 into pJG4-6 to make pJG4-6Cdi1; in this case, Cdi1 was expressed as a fusion protein containing, at its amino terminus, a methionine and the hemagglutinin epitope tag. The oligo(dT)-primed activation-tagged cDNA expression library, whose construction will be described elsewhere, was made from RNA from proliferating HeLa cells grown in 5% serum on plates to 70% confluence using a modification of the procedure described by Gubler and Hoffman (1993) and Huse and Hansen (1988) (J. G., unpublished data). The library contains 9.6×10^6 individual members, >90% of which contain a cDNA insert whose average size ranges between 1 kb and 2 kb; 25% and 33% of its members express fusion proteins.

Cdk Interactor Hunt

We began with a EGY48-p1840-*lexA-Cdc2* strain which contained *LexAop-LEU2* and *LexAop-lacZ* reporters and the LexA-Cdc2 bait. We introduced the library into this strain according to a variation of the procedure of Schiestl and Gietz (1983) (R. Finley, unpublished data). We recovered 1.2×10^7 transformants on glucose-Ura-His-Trp plates, scraped them, suspended them in approximately 20 ml

of 65% glycerol, 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and stored the cells in 1 ml aliquots at -80°C. We determined plating efficiency on galactose-Ura-His-Trp after growing 50 μ l of cell suspension for 5 hr in 5 ml of YP medium, 2% galactose. For the selection, about 2×10^7 galactose-viable cells were plated on four standard circular 10 cm galactose-Ura-His-Trp plates after galactose induction. After 4 days at 30°C, 412 *LEU*⁺ colonies appeared. These were collected on glucose-Ura-His-Trp master plates and retested on glucose-Ura-His-Trp-Leu, galactose-Ura-His-Trp-Leu, glucose-X-Gal-Ura-His-Trp, and galactose-X-Gal-Ura-His-Trp plates. Of these, 55 colonies showed galactose-dependent growth on Leu media and galactose-dependent blue color on X-Gal medium. We rescued plasmid DNAs from these colonies (Hoffman and Winston, 1987), introduced them into *E. coli* K8, and collected transformants on Trp-ampicillin plates. We classified library plasmids by restriction pattern on 1.8% agarose, 0.5 \times Tris-borate-EDTA gels after digestion with EcoRI and XhoI and either AluI or HaeIII. We reintroduced those plasmids from each map class that contained the longest cDNAs into EGY48 derivatives that contained a panel of different baits. Proteins encoded by each of the three cDNA classes interacted specifically with the LexA-Cdc2 bait, but not with other LexA fusion proteins (Figure 2).

Mammalian Expression

We grew HeLa cells on plates and transfected them (Ausubel et al., 1987–1993) either with a DNA copy of pBNCdi1 (J. G., unpublished data), a derivative of a neo resistance retroviral expression vector (Morgenstern and Land, 1990) that directs synthesis of native Cdi1 under the control of the Moloney murine leukemia virus promoter, or with vector alone. Cdi1 expression did not diminish the number of G418-resistant cells recovered. We rescued individual (about 20) clones from each transfection and grew them on plates in Dulbecco's modified Eagle's medium plus 10% calf serum. We resuspended cells from four Cdi1-transfected and four control-transfected clones in 225 μ l of 30 μ g/ml trypsin in 3.4 mM citrate, 0.1% Nonidet P-40, 1.5 mM spermine, and 0.5 mM Tris and incubated them on a rotator for 10 min at room temperature. We then added 188 μ l of 0.5 mg/ml of trypsin inhibitor and 0.1 mg/ml RNAase A, vortexed the suspension, added 188 μ l of 0.4 mg/ml of propidium iodide and 1 mg/ml spermine, and incubated the samples for 30 min at 4°C. For each sample, we analyzed 10,000–20,000 events in a Becton-Dickinson fluorescence-activated cell sorter using the CellFIT cell cycle analysis program (version 2.01.2).

Cdi1 and Cdi1-C140S

We made the Cdi1 Cys-140 to Ser mutation (Cdi1-C140S) by polymerase chain reaction with overlapping primers and Vent polymerase, essentially as described (Hanes and Brent, 1991), changing TGC at position 140 to TCT. Mutagenesis was verified by sequencing the entire mutant gene. We introduced EcoRI-XhoI fragments containing the wild-type and C140S coding sequences into a modified pGEX2T vector and expressed and purified the GST fusion proteins essentially as described (Ausubel et al., 1987–1993). We performed pNPP assays on these proteins in 500 μ l of 50 mM Tris-HCl (pH 7.2), 2 mM EDTA, 0.1% (v/v) β -mercaptoethanol, 20 mM pNPP (Sigma) containing the indicated amount of protein at 30°C. After 2 hr, we stopped the reactions by adding 50 μ l of 5 M NaOH and measured OD₄₀₅. For peptide assays, we labeled Raytide (Oncogene Science), at its tyrosine residue with [γ -³²P]ATP and c-Src tyrosine kinase (Oncogene Science) as described by the supplier. For phosphatase assays, we used 10⁵ cpm of radioactive [³²P]tyrosine-Raytide in 50 μ l reactions as described in Figure 5 and by Streuli et al. (1990).

Antibodies and Immunoprecipitation

Rabbit polyclonal antibody to GST-Cdi1 was produced by East Acres Biologicals, Incorporated (Southbridge, Massachusetts) according to standard methods (Harlow and Lane, 1988). Specificity of the antiserum was demonstrated by immunoprecipitation of in vitro translated Cdi1 (TNT-coupled reticulocyte lysate system, Promega). GST-Cdi1 blocked precipitation of this labeled product. Programmed lysate (5 μ l) was separately precipitated with 10 μ l of Cdi1 antiserum and 10 μ l of Cdi1 pre-immune serum in 500 μ l of phosphate-buffered saline in the presence or the absence of an excess (20 μ g) of GST-Cdi1 protein. Metabolic labeling and precipitations from subconfluent HeLa cells were performed essentially as described in Harlow and Lane

(1988) except that cells were lysed in 250 mM NaCl, 50 mM HEPES, KOH (pH 7.5), 5 mM EDTA, 0.1 mM NaVO₃, 50 mM NaF, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptin, 25 µg/ml pepstatin for 30 min at 4°C. In control precipitations, GST-Cdk1 blocked precipitation of Cdk1 and associated proteins. Pellets were run on 11% SDS protein gels that were analyzed either on a Molecular Dynamics image analyzer or by fluorography. For V8 mapping, bands were excised and partially digested in the gel with *Staphylococcus aureus* V8 protease as in Harlow and Lane (1988). Products, together with those resulting from digestion of in vitro translated Cdk2 and Cdc2, were separated on 17.5% SDS-polyacrylamide gels, dried, and visualized on the image analyzer.

Acknowledgments

We are very grateful to those named in the text for gifts of reagents and to Andrew Mendelsohn, Russ Finley, Joanne Kamens, Steve Hanes, Matt Meyerson, Emma Lees, John Little, Michaela Pagano, Guilio Draetta, Josh Kaplan, Robin Wharton, Stanley Fields, Jun Ma, and Richard Treisman for useful discussions, comments, or both on the manuscript. J. G. was supported by the Human Frontier Science Project (HFSP), E. G. by the National Institutes of Health, and R. B. by the Pew Scholars program and by an American Cancer Society faculty research award. Work was supported by the HFSP.

Received August 24, 1993; revised October 20, 1993.

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GenBank Accession Number

The number for the sequence reported in this paper is U02681.

Mxi1, a Protein That Specifically Interacts with Max to Bind Myc-Max Recognition Sites

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Summary

We used the interaction trap to isolate a novel human protein that specifically interacts with Max. This protein, Mxi1 (for Max interactor 1), contains a bHLH-Zip motif that is similar to that found in Myc family proteins. Mxi1 interacts specifically with Max to form heterodimers that efficiently bind to the Myc-Max consensus recognition site. When bound to DNA by a LexA moiety in yeast, Mxi1 does not stimulate transcription. *mxi1* mRNA is expressed in many tissues, and its expression is elevated in U-937 myeloid leukemia cells that have been stimulated to differentiate. These facts are consistent with a model in which Mxi1-Max heterodimers indirectly inhibit Myc function in two ways: first, by sequestering Max, thus preventing the formation of Myc-Max heterodimers, and second, by competing with Myc-Max heterodimers for binding to target sites.

Introduction

Members of the Myc protein family are involved in the formation of many cancers; in model systems their heightened expression can induce oncogenic transformation (for reviews see Cole, 1986; Lüscher and Eisenman, 1990), induce apoptosis (Evan et al., 1992; Shi et al., 1992), and block differentiation (Freytag, 1988; Miner and Wold, 1991). Of the Myc proteins, the best-studied are probably the *c-myc* and *v-myc* products (c-Myc and v-Myc). These proteins are localized to the nucleus (Abrams et al., 1982; Heaney et al., 1986; Stone et al., 1987) and can activate transcription when brought to DNA by heterologous DNA-binding domains (Lech et al., 1988; Kato et al., 1990; Golemis and Brent, 1992). These proteins contain an activation domain in their amino terminus, the integrity of which is correlated with their ability to cause oncogenic transformation (Kato et al., 1990; Barrett et al., 1992; E. A. Golemis et al., submitted). As do all Myc proteins, c-Myc and v-Myc contain a conserved structure, the basic region helix-loop-helix leucine zipper (bHLH-Zip) (reviewed in Vinson and Garcia, 1992), which directs oligomerization with other proteins and DNA recognition (Dang et al., 1989; Blackwell et al., 1990; Halazonetis and Kandil, 1992). These facts suggest that the biological function of c-Myc and v-Myc might depend on their ability to bind specific sequences and activate transcription.

Recently, two proteins have been isolated that form heterodimers with Myc proteins: Max, a human protein, and Myn, its murine homolog (Blackwood and Eisenman, 1991; Prendergast et al., 1991). Max protein and mRNA are expressed in all tissues in which c-Myc is expressed, and in some, including the adult brain, in which c-Myc is not. Two different forms of Max are encoded from differently spliced transcripts, a 151 amino acid protein (here called Max₁₋₁₅₁ or simply Max) and a larger form (here called Max₁₋₁₆₀) that contains an additional 9 amino acids at the amino terminus of the basic region. Max is localized to the cell nucleus, possibly owing to a nuclear localization sequence at its carboxyl terminus (Kato et al., 1992). Max has a longer half-life than c-Myc (>24 hr versus 30 min) (Blackwood et al., 1992a, 1992b; Hann and Eisenman, 1984; Lüscher and Eisenman, 1988). Like the Myc proteins, Max contains a bHLH-Zip motif. In Max, this motif allows Max to dimerize with itself and to form heterodimers with other members of the Myc family (Blackwood and Eisenman, 1991; Blackwood et al., 1992a, 1992b; Kato et al., 1992), but it does not direct interaction with other known bHLH-Zip proteins (Blackwood and Eisenman, 1991).

Max-Max homodimers and c-Myc-Max heterodimers bind tightly to a consensus CACGTG sequence (Blackwood and Eisenman, 1991; Prendergast et al., 1991). This fact strengthened the hypothesis that transcription regulation is important to Myc function; this idea has recently received additional support from the finding that in mammalian cells and in yeast, DNA-bound c-Myc-Max heterodimers activate transcription, while Max-Max homodimers do not (Kretzner et al., 1992; A. S. Z. and R. B., submitted). Because most c-Myc in vivo is thought to be associated with Max, and because c-Myc-Max heterodimers bind CACGTG sites more tightly than c-Myc-c-Myc homodimers, it appears likely that one function of Max is to facilitate the binding of c-Myc to these sites. It is also possible that association with Max modulates the regulatory activity of the c-Myc gene; consistent with this idea, we have recently shown that Max is transcriptionally inert but that its association with c-Myc greatly potentiates the strength of the Myc activation function (A. S. Z. and R. B., submitted). Myc-dependent transcription is also likely to be modulated by other cellular proteins, including oncoproteins, that change the availability of c-Myc or Max by affecting their expression or that alter c-Myc and Max DNA binding (Berberich and Cole, 1992), oligomerization, or transcription regulatory functions by altering their phosphorylation states (reviewed in Lüscher and Eisenman, 1990; Blackwood et al., 1992a, 1992b).

A great deal of circumstantial evidence suggests that control of Myc function may be important during differentiation. As mentioned, heightened expression of Myc in murine erythroleukemia cells and myocytes can block differentiation (Freytag, 1988; Miner and Wold, 1991), and it is clear that differentiation in model systems is often accom-

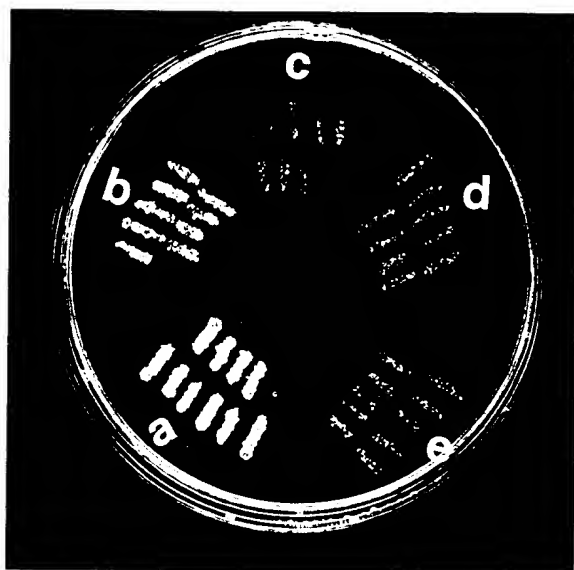


Figure 1. Activation by Interaction of Mxi1 with Different Protein Baits. Individual colonies of EGY48 yeast cells that contained tagged Mxi1 (the invariant activation tagged moiety of pJG4-5 [J. Gyuris, E. A. Golemis, and R. B., submitted, fused to Mxi1 residues 10–220]) and that also contained LexA–Max (a), LexA–c-Myc–C term (b), LexA–Cdc2 (c), LexA–FUS3 (d), and LexA–bicoid (e) were streaked with toothpicks onto a glucose–Ura⁺ His⁺ Trp⁺ master plate, and then replica plated onto a galactose–Ura⁺ His⁺ Trp⁺ Leu⁺ plate. Interaction results in activation of the *LexAop–LEU2* reporter and in growth in the absence of leucine. Mxi1 causes strong growth in the presence of the Max bait, weak growth in the presence of the c-Myc–Cterm bait, and no growth with Cdc2, FUS3, and bicoid baits.

panied by reductions in the amount of *c-myc* mRNA. For example, U-937 myeloid leukemia cells can be induced to differentiate into monocytes by treatment with TPA, as measured by withdrawal from the cell cycle and appearance of a number of monocytic markers, including expression of the *c-fms* proto-oncogene message (Hass et al., 1991). Differentiation is accompanied by a loss of detectable *c-myc* mRNA. In this system continued maintenance in culture results in eventual loss of the monocytic markers and resumption of proliferation; this “retrodifferentiation” is accompanied by a resumption of *c-myc* mRNA expression (Gunji et al., 1992).

In this work, we searched for proteins that interacted with Max. We undertook this study for two reasons. First, although the isolation of Max has provided insight into c-Myc function, it appeared possible that a complete understanding of the relevance of Max to the function of Myc would require isolation of the complete set of proteins that might affect its activity or availability. Second, in the nervous system, and in many cell lines, the apparent concentration of Max is much greater than that of any known Myc protein (E. Blackwood, personal communication), which suggested that Max in these cases might be available to form oligomers with other proteins that might affect its activity or amount.

We describe the use of a recently developed yeast genetic selection, the interaction trap (J. Gyuris, E. A. Golemis, and R. B., submitted), to isolate and characterize

Mxi1, a human protein that forms tight heterodimeric complexes with Max. Mxi1 contains a bHLH–Zip sequence that allows it to interact specifically with Max and bind to Myc–Max consensus sites, but in yeast assays the protein does not stimulate transcription. Its sequence indicates that Mxi1 clearly belongs to the same family as Mad, another protein that interacts with Max and is described in an accompanying paper (Ayer et al., 1993). Expression of *mxi1* mRNA is greatly elevated in U-937 cells that are induced to differentiate. These facts are consistent with a simple model in which Mxi1 and Mad indirectly regulate c-Myc function by sequestering Max.

Results

Isolation of Mxi1

We selected cDNAs that encoded Mxi1 from a HeLa cell library by the interaction trap (J. Gyuris, E. A. Golemis, and R. B., submitted). In this technique, which was developed from a suggestion made by Fields and Song (1989), a library that conditionally expresses cDNA-encoded proteins fused to an epitope tag, a nuclear localization sequence, and an acidic transcription activation domain is introduced into a special yeast strain. This strain, EGY48, contains a plasmid that directs the synthesis of a transcriptionally inert LexA derivative (the “bait,” in this case LexA–Max) and two different reporter genes, the transcription of which is stimulated if the library encoded protein complexes with the bait. One reporter, an extremely sensitive *LexAop–LEU2* gene, allows growth in the absence of leucine; the other, one of a series of *LexAop–lacZ* genes of different sensitivities to activators, directs the synthesis of β -galactosidase (J. Gyuris, E. A. Golemis, and R. B., submitted).

As described in Experimental Procedures, we performed the selection with EGY48 that contained LexA–Max and that also contained pJK103, a medium-sensitive *LexAop–lacZ* reporter (Kamens et al., 1990). We plated a pool of cells that contained 7.5×10^5 primary library transformants at a multiplicity of 5 onto galactose–Leu⁺ selection plates, and picked the first 100 Leu⁺ colonies that appeared. Of these, 80 that showed unambiguous galactose-dependent blue color on X-gal medium were analyzed further (see Experimental Procedures). As expected, restriction mapping and partial sequence analysis showed that many of these (62) encoded human c-Myc. From the remainder, we selected for further characterization the restriction map class (2 members) whose products gave the strongest activation with the LexA–Max bait. We called the gene encoded by these *mxi1* (for Max interactor 1) and its protein product Mxi1. We tested the specificity of the Mxi1–Max interaction by testing the ability of Mxi1 to interact with a panel of different baits. Visual inspection of activation of a *LexAop–LEU2* reporter on galactose–Leu⁺ plates showed that Mxi1 interacted strongly with LexA–Max, detectably with LexA–c-Myc–Cterm, a bait that contained the c-Myc bHLH–Zip, but not with LexA–Cdc2, LexA–FUS3, and LexA–bicoid (Figure 1).

Figure 2. Sequence of *mx11* cDNA and Protein

nucleotide match (allowing one gap) with one of the

Primary Sequence

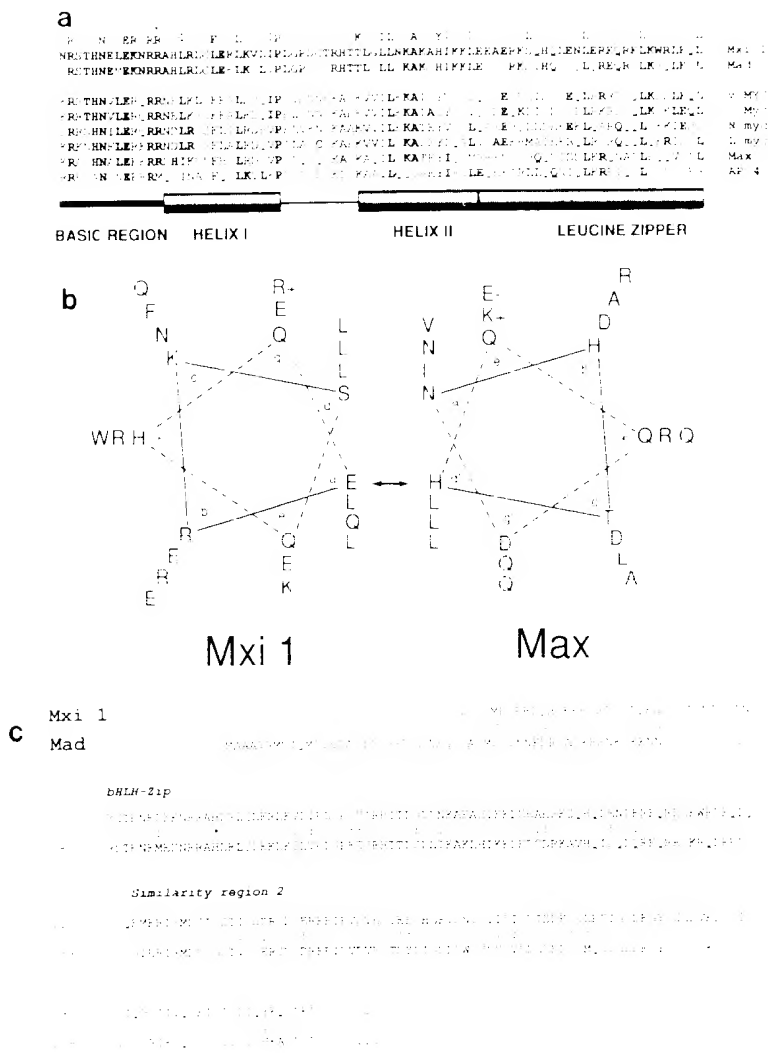
Primary Sequence
We sequenced the partial *mx1* open reading frame on the library plasmid. As expected, it was fused in-frame to the invariant amino-terminal moiety of the vector-encoded protein. We used this cDNA to isolate a 2.4 kb cDNA from a commercially available HeLa cDNA library. The sequence of this cDNA revealed a 220 amino acid open reading frame with 315 5' flanking nucleotides and 1440 3' nucleotides (Figure 2).

The *mx11* open reading frame contained a bHLH-Zip region between amino acids 32–112 that was very similar in sequence to the bHLH-Zip found in Myc family proteins and in Max (Figure 3a). Of 13 amino acids in the Mx11 basic region 8 are identical to those in human c-Myc, including the arginine at position 44, which contacts the CG core of the c-Myc–Max consensus binding site (Halazonetis and Kandil, 1992; Dang et al., 1992). There is also substantial sequence similarity outside of the basic region: the Mx11 HLH is a near match to the HLH consensus and, as in Myc family proteins (Beckmann and Kadesch, 1991), HLH helix II is fused to the leucine zipper so that the periodic hydrophobic heptad repeat in the zipper continues into the C-terminus of helix II (Figure 3a). We projected the Mx11 and Max leucine zippers onto a helical wheel (Figure 3b). As Myc proteins, Mx11 contains an acidic residue (Glu-87)

at the a position that might interact favorably with the histidine found at the d' position of Max. Furthermore, as do Myc proteins, Mxi1 contains charged residues at the g position that might favorably interact with charged residues in the e' position of Max.

Outside of the bHLH-Zip, Mxi1 shows no similarity to previously identified proteins. The sequence does not reveal a classical nuclear localization signal, although Arg-13, Arg-14, Lys-80, and Lys-81 might conceivably constitute a bipartite localization signal (Dingwall and Laskey, 1991). Mxi1 contains an acidic stretch at residues 131 to 169 (15 residues are glutamic or aspartic acid, predicted net charge of -14). However, projection of this stretch of acidic amino acids onto a helical wheel showed that the putative α helix formed by this stretch did not show the amphipathicity sometimes associated with putative α helices formed by functional activation domains (Ruden et al., 1991). The Mxi1 3' noncoding region contained a stretch with substantial similarity to an expressed sequence tag found on a cDNA isolated from human brain (Adams et al., 1992) (see the Figure 2 legend).

Mxi1 does show pronounced similarity to another Max-interacting protein, Mad, which is described by Eisenman and his coworkers in an accompanying paper (Ayer et al., 1993). We view the similarity as consisting of two regions,



bHLH-Zip at Mxi1 residues 33–112 and similarity region 2 at Mxi1 residues 116–187. Similarity region 2 contains a stretch of amino acids (Mxi1 residues 127–150) similar to a stretch of amino acids of unknown function found in Myc family proteins (residues 246 to 268 in human c-Myc).

the bHLH-Zip (Figures 3a and 3c) and a second region C-terminal to this motif, which we call similarity region 2, that in Mxi1 is separated from the bHLH-Zip by a 4 amino acid connector (Figure 3c). The Mxi1 bHLH-Zip region is more similar to that of Mad than it is to any other bHLH-Zip; the basic region and helix 1 are almost identical, consistent with the fact that Mad also recognizes Myc-Max sites, and the helix 2 and leucine zipper are also very similar to that of Mad: Mad even possesses an acidic residue (Asp-112) in the a position of the leucine zipper helix homologous to the Glu-87 in Mxi1. In both proteins, similarity region 2 (Mxi1 residues 115 to 189) is rich in serines and acidic residues and contains (residues 127 to 150) a stretch of amino acids similar to a region of unknown function in Myc family proteins (see the Figure 3c legend). Mad also contains an amino-terminal extension (Figure 3c) that is not found in Mxi1.

Association with Other Proteins

We further explored the specificity of the Mxi1–Max interaction by repeating our initial interaction assays with acti-

Figure 3. Speciated Fractions of the Metal Ion

(b) The Mx1 bHLH-Zip. Shown is a comparison of the pHLH-Zip region from Mx1 (residue 32 to residue 112) to pHLH-Zip regions in other proteins. The Mx1 bHLH-Zip is shown in bold. Residues common to at least four members of the set (Mx1, Mad, c-Myc, Max, avian v-Myc, human n-Myc and 1-Myc, and AP 4) are shown by stippling. Residues shared by other members of the set with Mx1 are shown in bold. The C-terminal arginine within the basic region, which in c-Myc specifies interaction with the CG core of the CACGTG recognition sequence, is shown with an asterisk (Hatzioannetis and Kandil, 1992; Dang et al., 1992). Highly conserved residues that are likely to be critical for the framework of the structural motif are shown above the Mx1 line in bold. Φ denotes hydrophobic amino acids. Sequences of the Myc family pHLH-Zip were taken from Benevise et al. (1990), Max from Blackwood and Eisenmann (1991), AF4 from Hu et al. (1993), and Mad from Aver et al. (1993).

(f) Leucine zippers of Mx1 and Max. The leucine zippers from Mx1 and Max are each shown projected onto a helical wheel as in O'Shea et al. (1989). Amino acids at the c' position of Mx1 and the e' position of Max are shown with their respective charges; the arrow indicates the glutamic acid at the amino terminus of the Mx1 leucine zipper (Mx1 position a), which is invariant in all Myc proteins, and the histidine opposite it in Max leucine zipper position d'.

(c) Comparison of Mx-1 and Mad- Sequences were aligned by the Genetics Computer Group (GAP program (Devereux et al., 1984). Solid lines between amino acids indicate identity at the corresponding position, two dots indicate a high degree of similarity, and one dot indicates less similarity. The overall similarity between Mx-1 and Mad is 79% with 59% identity. Two regions of highest similarity are identified: the

vation tagged Mxi1, a series of more closely related baits, and two reporters, the *LexAop-LEU2* reporter in EGY48 and the *LexAop-lacZ* reporter pJK103. These reporters exhibit differential sensitivity to activators: the *LexAop-LEU2* reporter is exquisitely sensitive, while the *LexAop-lacZ* reporter is less so; expression of their phenotypes provides a measure of the strength of the interaction. Results are given in Table 1. With the less sensitive reporter, we only detected significant interaction between Mxi1 and a LexA-Max bait; any other interactions were below the level we consider significant (Golemis and Brent, 1992). With the more sensitive reporter, we detected a strong interaction between Mxi1 and Max and a weak interaction between Mxi1 and derivatives of c-Myc and n-Myc that contained bHLH-Zip. Mxi1 exhibited basal activation activity below the level we consider significant (see below) and did not show significant interactions with itself. We then confirmed the specificity of the Mxi1-Max interaction by immunoprecipitation experiments with *in vitro* translated Mxi1, Max, c-Myc, and n-Myc (A. S. Z., M. Billard, and R. Bernard, unpublished data). As judged by immuno-

Table 1. Interaction of Mxi1 with Related Proteins

Fusion Protein	Glucose		Galactose	
	Leu ⁻	β -galactosidase	Leu ⁻	β -galactosidase
LexA-c-Myc-Cterm	-	<2	-	10
LexA-Max	-	<2	+++	120
LexA-Mxi1	-	10	-	10
LexA-Hairy	-	<2	-	<2
LexA-id	-	<2	-	<2
LexA-n-Myc-bHLH-Zip	-	<2	+	8
LexA-Da	-	<2	-	<2

Plasmids that directed the synthesis of activation tagged Mxi1 and the listed baits were introduced into EG 48. In addition to the *LexAop-LEU2* reporter, the strain also harbored pJK103, a medium-sensitive *LexAop-lacZ* reporter. Basal activation by the baits was monitored by observation of the Leu⁻ phenotype on glucose Leu⁻ plates, and by measurement of β -galactosidase levels in liquid cultures grown on glucose. Interaction with Mxi1 was monitored by comparison of these phenotypes with those when cells were grown on the equivalent galactose medium, under which conditions Mxi1 was expressed. β -Galactosidase levels were performed in duplicate on three independent isolates; the average values given here.

precipitation with anti-Max antiserum, Mxi1 was associated with Max, but, at the lower protein concentrations in these experiments, Mxi1 did not associate with c-Myc or n-Myc (not shown; see Discussion).

Site Recognition

The sequence of the Mxi1 bHLH-Zip suggested that complexes between Mxi1 and Max might bind the consensus Myc-Max recognition site. We tested this idea by assaying the ability of combinations of glutathione S-transferase (GST)-Mxi1₂₂₂, purified Max₁₅₁, and purified c-Myc bHLH-Zip₃₄₂₋₄₃₉ to retard the electrophoretic mobility of an oligonucleotide that contained the CACGTG Myc-Max consensus site (Halazonetis and Kandil, 1991). These results are shown in Figure 4. As expected, purified c-Myc bHLH-Zip caused the formation of a very small amount of retarded complex (Figure 4, lane 1, complex c), purified Max caused the appearance of a larger amount of retarded complex of different mobility (lane 2, complex b), purified GST-Mxi1 did not give detectable amounts of retarded complex (lane 3), which we attribute to its inability to interact with itself (Table 1), and GST-Mxi1 and c-Myc bHLH-Zip did not form appreciable amounts of a new complex (lane 5). However, addition of GST-Mxi1 to Max resulted in the appearance of a complex of altered mobility (complex a, lane 6); the amount of this complex was increased with increasing amounts of GST-Mxi1 (lanes 7 and 8). This complex contained both Mxi1 and Max; treatment of the binding mixes with glutathione-Sepharose removed this complex quantitatively from the binding mix (lane 9), and treatment of the binding reactions with anti-Max antiserum resulted in the disappearance of most of the Mxi1-Max-DNA complex and its apparent supershift to a much less mobile DNA complex (lane 10). These results demonstrate that Mxi1 and Max complexes bind Myc-Max recognition sites.

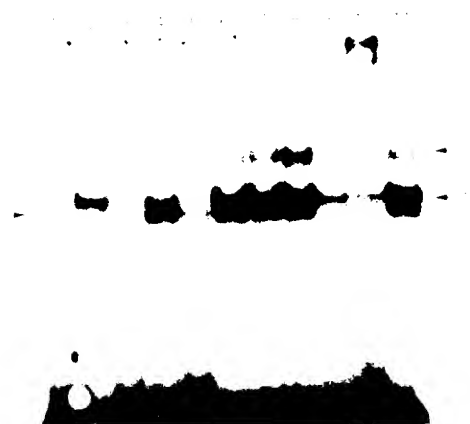


Figure 4. Sequence-Specific DNA Binding by Mxi1 and Max

Binding assays used bacterially produced GST-Mxi1, full-length Max, and c-Myc bHLH-Zip (amino acids 342-439). The labeled oligonucleotide contained a consensus Myc-binding site. Binding reactions (20 μ l) containing the indicated proteins and other reagents were performed, run on a 5% polyacrylamide gel, and subjected to autoradiography as described (Experimental Procedures). a, b, and c denote specific oligonucleotide-containing complexes. Lane 1, 300 ng of c-Myc bHLH-Zip; lane 2, 10 ng of Max; lane 3, 10 ng of GST-Mxi1; lane 4, 10 ng of Max, 300 ng of c-Myc bHLH-Zip; lane 5, 300 ng of Max, 300 ng of c-Myc bHLH-Zip; lane 6, 10 ng of Max, 10 ng of GST-Mxi1; lane 7, 10 ng of Max, 20 ng of GST-Mxi1; lane 8, 10 ng of Max, 50 ng of GST-Mxi1; lane 9, 10 ng of Max, 50 ng of GST-Mxi1, pretreated with 10 μ l of 50% v/v glutathione-Sepharose beads, supernatant loaded on gel; lane 10, 10 ng of Max, 50 ng of GST-Mxi1, 1 μ l of anti-Max antiserum; lane 11, 10 ng of Max, 50 ng of GST-Mxi1, 1 μ l of control antiserum.

Transcription Activation

We assayed the ability of Mxi1 to stimulate transcription when expressed in yeast as a LexA derivative and bound to LexA operators in the p1840 *LexAop-lacZ* reporter. Table 2 shows that, unlike c-Myc but like Max (Lech et al., 1988; Golemis and Brent, 1992; A. S. Z. and R. B., submitted), in this assay Mxi1 is transcriptionally inert. When transcription is assayed on the more sensitive *LexAop-lacZ* reporter pJK103 (not shown), Mxi1 displays basal transcription well below the level that can be considered significant (Golemis and Brent, 1992). While some activation domains that function in higher cells, such as the glutamine-rich regions of SP1, the *Drosophila* zeste product, and the proline-rich domains in CTF, do not function in yeast (Pugh and Tjian, 1990; C. Besmond and R. B., unpublished data), our experience has been that acidic activation domains usually do, and this yeast result thus suggests that the acidic portion of Mxi1 is not a functional activation domain.

mRNA Expression

To determine in which tissues *mxi1* mRNA was expressed, we probed a Northern blot containing 2 μ g of poly(A) RNA from different human tissues with *mxi1*. This experiment revealed that *mxi1* mRNA was expressed in every tissue tested, with the highest levels in heart, brain, and lung (Figure 5). Because Mxi1 was isolated as a Max partner, we monitored the expression of *max* mRNA as well; it closely mirrored that of *mxi1* (Figure 5). Northern analysis of *mxi1* and *max* mRNAs from serum-starved and serum-

Table 2. Activation in Yeast by Mxi1, Max, and c-Myc

Protein	p1840
LexA-c-Myc	12
LexA-Max	2
LexA-Mxi1	2

Plasmids directing the synthesis of LexA derivatives of the intact proteins were introduced into EGY40/p1840, and β -galactosidase assays were performed on cultures of independent transformants as described in the text.

stimulated WI38 human lung fibroblasts showed that both messages were expressed at high levels, but were not induced by serum stimulation (not shown). Similar analysis of mRNA levels in populations of HeLa cells that were synchronized by a double thymidine block (Rao and Johnson, 1970) showed that *mxi1* and *max* were expressed at easily detectable levels, but that their amounts did not vary with the stage of the cell cycle (not shown).

We then examined the expression of *mxi1*, *max*, and *c-myc* in mRNA extracted from differentiating cells of myeloid lineages. For these experiments, we used RNA (a generous gift of Ralph Hass and Donald Kufe) derived from two different premonocytic cell lines, U-937 and HL-60. These lines are believed to correspond to different stages of monocytic development; U-937 is thought to be derived from a more determined cell type than HL-60. These lines can be induced to differentiate by a number of agents (reviewed in Harris and Ralph, 1985); when so treated, U-937 cells differentiate along the monocytic pathway, while HL-60 cells differentiate into monocytes or granulocytes depending on the inducing agent. We measured the expression of these messages in myeloid cells for two reasons. First, because constitutive expression of c-Myc can block their differentiation (Freitag, 1988; Kume et al., 1988); second, because down-regulation of *c-myc* mRNA levels occurs upon differentiation (Watanabe et al., 1985; Gujji et al., 1992), we searched for changes in the levels of *myc*-related messages that might also occur during the differentiation of these cells.

As shown in Figure 6, TPA, the strongest inducer of U-937 differentiation, induces *mxi1* mRNA by a factor of about 20 (lanes 1–5). To verify that this induction was a consequence of differentiation, rather than a simple transcriptional response to TPA itself, we examined the *mxi1* message in RNA extracted from cells that were induced to differentiate with a number of other agents, including dimethylsulfoxide, retinoic acid, vitamin D3, tumor necrosis factor α , and okadaic acid. Treatment with these agents also induced *mxi1* mRNA expression (not shown); the amount of *mxi1* mRNA in cells treated with these agents paralleled their potency as inducers of differentiation (as determined by changes in morphology, and induction of the *c-fms* message) (not shown). In U-937 cells, *max* mRNA was low and its expression was not affected by treatment with TPA (Figure 6). *c-myc* mRNA, as expected (Gujji et al., 1992), was induced briefly, and then returned to a very low level (Figure 6). By contrast, in HL-60 cells, the basal level of *mxi1* and *max* mRNA was much higher

(lane 6). This level was not elevated in RNA from cells that were induced to differentiate with retinoic acid, although, as expected, the amount of *c-myc* mRNA was greatly reduced (lanes 7 and 8) (Watanabe et al., 1985).

Discussion

We used the interaction trap (J. Gyuris, E. A. Golemis, and R. B., unpublished data), an implementation of a suggestion made by Fields and Song (1989), to isolate Mxi1, a protein that interacts specifically with Max. The *mxi1* cDNA spans 2417 bp. Within it, there is an open reading frame that encodes a 220 amino acid protein, followed by a very large 3' untranslated region. The Mxi1 protein predicted from this open reading frame contains an acidic stretch at the carboxyl terminus but lacks a conspicuous nuclear localization signal. Mxi1 contains a bHLH-Zip motif at its amino terminus. The sequence of the Mxi1 bHLH-Zip is extremely similar to that of Myc family proteins; the basic region is identical in a residue these proteins use to contact DNA. Helical wheel projections suggest that the Max-Mxi1 interaction may depend on a favorable ionic interaction between the leucine zippers of the two proteins. Mxi1 is quite similar in sequence to another Max interactor, Mad, described in an accompanying paper (Ayer et al., 1993). Mad shows two regions of similarity to Mxi1, the bHLH-Zip, and a second stretch, similarity region 2, which we imagine has a conserved structure but whose function is now unknown (Figure 3c); Mad also contains a pronounced dissimilarity, a 33 residue extension at its amino terminus that has no equivalent in Mxi1 (Figure 3c).

We tested the function of Mxi1 in three ways. First, we used the interaction trap to test further the specificity of

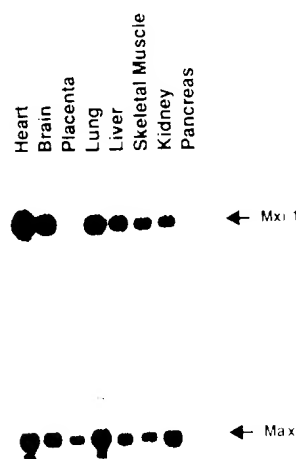


Figure 5. *mxi1* and *max* mRNA in Different Tissues

Figure shows a Northern blot of mRNA from different human tissues that was probed with *mxi1*, then stripped and reprobed with *max* as described (Experimental Procedures). Each lane contains 2 μ g of poly(A)⁺ mRNA from the indicated tissues. *mxi1* mRNA runs with an apparent mobility of 3.2 kb, and *max* runs with an apparent mobility of 2 kb.

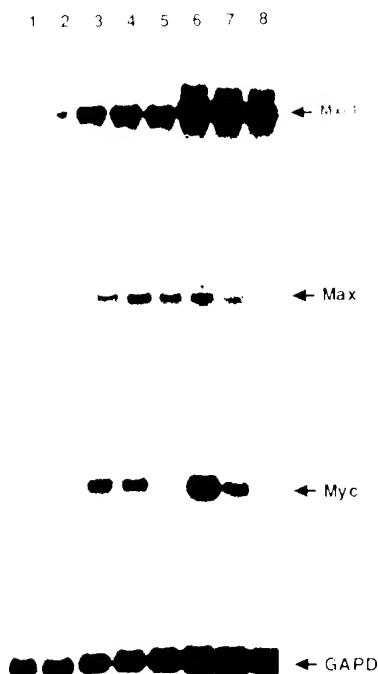


Figure 6. *mxi1*, *max*, and *myc* mRNA during Differentiation

RNA was isolated from U-937 and HL-60 cells at different times after they were induced to differentiate with TPA (U-937) or retinoic acid (HL-60). RNA (20 μ g) from cells from each time point was run on a gel and blotted onto a nylon membrane, which was probed successively with *mxi1*, *max*, *c-myc*, and human GAPD as described (Experimental Procedures). Lane 1, U-937, untreated; lane 2, U-937, 1 hr after TPA induction; lane 3, U-937, 3 hr after TPA induction; lane 4, U-937, 6 hr after TPA induction; lane 5, U-937, 12 hr after TPA induction; lane 6, HL-60, uninduced; lane 7, HL-60, 24 hr after retinoic acid induction; lane 8, HL-60, 72 hr after retinoic acid induction.

the interaction between Mxi1 and Max. We found that Mxi1 interacted strongly with Max, weakly with c-Myc, and not at all with other related bHLH-Zip, bHLH, and bZIP proteins, including Mxi1 itself. We confirmed this result by immunoprecipitation experiments showing that in vitro translated Mxi1 interacted specifically with Max. Second, we performed gel retardation experiments that suggest that Max-Mxi1 heterooligomers recognize consensus Myc-Max-binding sites. Third, we performed yeast transcription experiments. In yeast, Mxi1 has almost no activation function. Since acidic activation regions that function in higher cells typically function in yeast (R. B., unpublished data; G. Gill and R. Tjian, personal communication), these experiments suggest that the acidic residues in similarity region 2 do not contribute to a mammalian activation domain.

Finally, in an effort to understand Mxi1 function in vivo, we examined the expression of its mRNA. *mxi1* mRNA is expressed in all tissues tested, including ones, such as the adult brain, in which *c-myc* is not expressed. The cells in these tissues are terminally differentiated. To explore whether Mxi1 expression might change during differentiation, we monitored the expression of *mxi1* mRNA in my-

eloid cell lines whose *c-myc* mRNA levels were known to depend on their differentiation state. We found that, in U-937 cells that were provoked to undergo differentiation, the expression of *mxi1* mRNA was strongly elevated at all times after induction, while expression of *max* and *c-myc* mRNA was elevated only slightly and at intermediate times. In HL-60 cells, which are derived from less determined cells, basal expression of *mxi1*, *max*, and *myc* mRNAs was high; when granulocytic differentiation was provoked by induction with retinoic acid, expression of *mxi1* and *max* mRNA was not greatly altered, while, as expected, expression of *c-myc* mRNA was strongly reduced.

As assayed by interaction trap and gel retardation assays, Mxi1 interacts strongly with Max. The sequence of Mxi1 suggests one possible explanation for this specificity: a favorable ionic interaction between the Mxi1 and Max leucine zippers. Ascribing an important function to this interaction seems reasonable given the contribution that favorable leucine zipper interactions can make to dimerization of another bHLH-Zip protein, c-Myc itself (Halazonetis and Kandil, 1991). Interaction trap experiments show that Mxi1 does not associate with itself, but do reveal a weak association between Mxi1 and c-Myc and between Mxi1 and n-Myc. These associations are only convincingly revealed by the very sensitive *LexAop-LEU2* reporter in EGY48; they are marginally detected by the *LexAop-lacZ* reporter carried on pJK103 and are not detected by less sensitive *LexAop-lacZ* reporters or immunoprecipitation experiments (not shown). We believe the weak interactions are only revealed because of the relatively high intranuclear concentrations of Mxi1 in these experiments (10^{-6} M in the yeast nucleus versus $<10^{-9}$ M in typical immunoprecipitation experiments with in vitro translated proteins). The yeast experiments illustrate two points: that interaction trap assays provide a simple way to examine lower affinity protein-protein interactions than those detected by typical experiments with in vitro translated proteins and that the use of different reporters of different sensitivities in these assays can provide a measure of the strength of the interactions detected. We believe that further refinements of the interaction trap will allow its use to determine relative interaction affinities quantitatively; for the moment, we can neither determine the relative affinities of Mxi1 and c-Myc for Max, nor, because the intranuclear concentrations of Mxi1 and Myc proteins in mammalian cells are not known, can we determine whether the weak Mxi-Myc associations are likely to be of biological significance.

This work and the accompanying paper (Ayer et al., 1993) show that Max and Myc family proteins are members of a larger group of interacting proteins, within which Mxi1 and Mad constitute a distinct subfamily of Max interactors that may also include other members that have not yet been identified. Since Mxi1 and Mad may be expressed in different cell types and may also differ in biochemical function, it is possible that understanding Myc family protein function in mammalian organisms may require identification and thorough study of all the proteins that comprise this interacting group. However, we have identified four

facts about Mxi1 that we believe are likely to be significant for such future understanding: its interaction with Max, its ability when complexed with Max to bind Myc-Max recognition sites, its lack of activation function in yeast, and its induction in differentiating monocytic cell lines. These facts suggest that Mxi1 may negatively regulate Myc function in two ways: first by sequestering the Max that Myc needs to bind its DNA site and, second, by forming inert complexes with Max that compete with transcriptionally active Myc-Max heterodimers for binding sites. According to this view, Mxi1 would antagonize Myc-dependent oncogenesis, and, when its synthesis was induced during differentiation, it would accelerate the loss of c-Myc activity caused by down-regulation of the *myc* mRNA.

Experimental Procedures

Standard manipulations of *Escherichia coli*, nucleic acids, and yeast were performed essentially as described (Ausubel et al., 1992; Guthrie and Fink, 1992). *E. coli* K-12 strain K08 *pyrF::Tn5*, *hsdR*, *leuB600*, *trpC9E30*, *lacD74*, *trpA*, *galK*, *hisB436* was used for the rescue of yeast plasmids as described (J. Gyuris, E. A. Golemis, and R. B., submitted).

Selection Strain

EGY48 MATa *trp1 ura3 his3 LEU2::pLexAop6-LEU2* was used as a host for all interaction experiments; it will be described in detail elsewhere (J. Gyuris, E. A. Golemis, and R. B., submitted). In the selection, it contained the reporter pJK103 (Kamens et al., 1990), which directs expression of a *GAL1-lacZ* gene from two high affinity ColE1 LexA operators (Ebina et al., 1983). This reporter presumably binds four LexA dimers; it is at least 5 times as sensitive to activation by LexA-containing proteins as is p1840 (Brent and Ptashne, 1984; J. Gyuris et al., unpublished data), although it is still not as responsive as the *LexAop-LEU2* reporter in EGY48.

Baits

All plasmids used to express the different baits were based on pL202Pl (Huden et al., 1991), which carries the HIS3⁺ marker and a 2 μ m replicator. All baits contained at their amino terminus the LexA DNA-binding domain and the C-terminal dimerization domain, which directs efficient operator occupancy by the bait (Golemis and Brent, 1992). LexA-Max contains the entire 151 residue form of the human Max protein (Blackwood and Eisenman, 1991), and LexA-c-Myc-Cterm (A. S. Z. and R. B., submitted) contains the carboxy-terminal 176 amino acids of human c-Myc, but lacks the activation domains (Kato et al., 1990; E. A. Golemis et al., submitted). Both were cloned as EcoRI-BamHI fragments into pL202Pl after polymerase chain reaction amplification (A. S. Z. and R. B., submitted); in these constructions no amino acids were introduced into the junction of these fusion proteins. LexA-FUS3 and LexA-Cdc2, which contain the entire yeast FUS3 and the human Cdc2 proteins, were as described (J. Gyuris, E. A. Golemis, and R. B., submitted). LexA-CLN3, which contains the entire yeast *CLN3* coding sequence, was a gift of Jenő Gyuris. LexA-bicoid, which contained residues 2–160 of the *Drosophila bicoid* gene product, was a gift from Russ Finley. LexA-n-Myc, containing the 102 C-terminal amino acids of the n-Myc protein, including the bHLH-Zip, LexA-Iid, which contained amino acids 64–133 of the human Iid protein, was a gift of Steve Sloan and Tom Kadash. LexA-Hary and LexA-Ca, which contained the full-length *Drosophila hairy* product and residues 485–761 of the *daughterless* product, was a gift of Ze'ev Paroush and David Ish-Horowitz.

Library

The oligodT₁₂-primed HeLa cDNA yeast expression library will be described elsewhere (J. Gyuris et al., unpublished data). The vector, pJG4-5, directs the synthesis of library-encoded proteins that carry, at their amino termini, the influenza virus HA1 epitope tag, the B42 acidic activation domain, and the SV40 nuclear localization signal.

Selection of Max Interactors

An interaction trap selection (J. Gyuris, E. A. Golemis, and R. B., unpublished data) was performed in EGY48 into which pJK103 and PL-Max had been introduced by transformation. This strain was maintained under selection for the URA3 and HIS3 markers and was transformed with the HeLa interaction library using a variation of the lithium acetate method of Ito et al. (1983) (R. Finley, personal communication). A total of 750,000 primary yeast transformants were selected on ten 25 \times 25 cm Ura⁺ His⁺ Trp⁺-glucose plates, scraped, pooled, and stored at -70°C. Plating efficiency was determined on Ura⁺ His⁺ Trp⁺-galactose plates and 5 colony-forming units per original transformant, approximately 4 \times 10⁵ cells, was plated on two standard Ura⁺ His⁺ Trp⁺-galactose plates. Three days later, colonies appeared; these were restreaked and tested on Ura⁺ His⁺ Trp⁺-gal-glucose and Ura⁺ His⁺ Trp⁺-gal-galactose. Plasmids from colonies that grew on Leu⁺ plates and turned blue on X-gal medium were isolated as described by Hoffman and Winston (1987) and introduced into K08 cells by electroporation. Library plasmids were selected by plating the transformation mix on 1 \times A glucose plates containing ampicillin, uracil, histidine, leucine, and thiamine (Miller, 1972), but lacking tryptophan.

cDNAs were analyzed first by restriction mapping using EcoRI, XhoI, and AluI enzymes and sorted into classes depending on their restriction map pattern. At least one representative cDNA from each class was partially sequenced by the dideoxy method using modified T7 DNA polymerase and a commercially available kit (US Biochemicals). The full-length cDNA clone for Mxi1 was isolated from a HeLa cDNA library in λ ZAPII (Stratagene, Inc.). A total of 1.5 \times 10⁶ plaques were screened using standard techniques (Ausubel et al., 1992). Three positive clones were isolated, of which the longest contained a cDNA insert of 2.4 kb. Following phagemid rescue the cDNA was completely sequenced using customized oligonucleotide primers and collapsed supercoiled DNA as template as above.

DNA Binding Assays

Gel retardation DNA binding assays were performed essentially as described in Papoulas et al. (1992). Purified Max and c-Myc bHLH-Zip (which contained residues 342–439 of human c-Myc), made from recombinant *E. coli*, were gifts of Ophelia Papoulas. GST-Mxi1 was made as follows: a fragment that contained the entire coding sequence of Mxi1 was amplified using polymerase chain reaction, subcloned into pGEX-2T (Pharmacia), and transformed into DH5 α cells. The fusion protein was induced and isolated as described (Smith and Corcoran, 1989). A double-stranded oligonucleotide, 5'-GGAAAGCAGACACGGTGGTCTGCTTCC-3', that contained the CACGTG consensus site was a gift from Thanos Halazonetis. Approximately 0.5 μ g of oligonucleotide was end-labeled with [γ -³²P]ATP. Binding assays contained the indicated concentrations of purified Max, c-Myc bHLH-Zip, GST-Mxi1, and 0.5 ng of the labeled DNA probe. Assays were performed in a volume of 20 μ l so that the concentration of binding site was 2.5 \times 10⁻¹¹ M, the concentration of GST-Mxi1 ranged from 3 \times 10⁻¹¹ M (1 μ l of Mxi1 in the binding reaction) to 1.5 \times 10⁻¹¹ M (5 μ l in the binding reaction), the concentration of Max was typically 5 \times 10⁻¹¹ M, and the concentration of c-Myc bHLH-Zip, which had been purified after renaturation after guanidium treatment of an insoluble protein pellet, was 2.5 \times 10⁻¹¹ M. In these assays, the poly(dI-dC) often used as a nonspecific competitor was substituted with 200 ng (1200 \times molar excess over labeled oligonucleotide) of a nonspecific single-stranded oligonucleotide (5'-GTAATGCATCCAGTTC-3') (as in Halazonetis and Handl, 1991). Binding was allowed to proceed for 20 min at room temperature in reaction buffer that contained 10 mM Tris (pH 7.4), 80 mM NaCl, 1 mM dithiothreitol, 5% glycerol. Where indicated, 1 μ l of either control or anti-Max antiserum was added to the reactions. Binding mixtures were run on a 5% polyacrylamide gel (29:1 acrylamide:bisacrylamide) that contained 0.5 \times TBE buffer with 0.01% NP-40 with running buffer that contained 0.5 \times TBE and 0.05% NP-40, after which gels were dried and autoradiographed.

RNA Isolation and Northern Blot Analysis

mRNA from differentiating U-937 and HL-60 cells was a gift of Rafi Hass and Donald Fufe. Cells were grown in RPMI 1640 containing 10% (for U-937) or 15% (for HL-60) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. U-937 cells were treated with 32 nM TPA (Sigma) and HL-60 cells

were treated with 1 μ M hydrochloric acid (5 g/ml) for the indicated times. Total RNA was isolated using the guanidine isothiocyanate-cesium chloride method (Chirgwin et al., 1979). RNA (20 μ g) from each time point was run on 1% agarose-formaldehyde gels and transferred to nylon membranes (Zeta Probe, Bio-Rad) ultraviolet cross-linked, and hybridized as described (Ausubel et al., 1992) to the following ³²P-labeled DNA probes: probe a, a 425 bp ϕ hoI- ϕ hoI fragment from pT28 corresponding to nucleotides 595 to 1910 of Mxi1; probe b, a 460 bp EcoRI-BamHI fragment from plasmid pSHmax (A. S. Z., unpublished data) carrying the full-length coding sequence of Max; probe c, a 1325 bp EcoRI-BamHI fragment from pSHmyc (A. S. Z., unpublished data) that contained the human c-Myc coding sequence; probe d, a 1268 bp PstII-PstII fragment that carried the full-length human glyceraldehyde phosphate dehydrogenase (GAPD) coding sequence (Tokunaga et al., 1987).

Acknowledgments

We are grateful to Erica Golemis and Russ Finley for plasmids and strains used in the interaction trap, to those named in the text for gifts of bait plasmids, to Marc Billard and René Bernards for help with immunoprecipitations and useful conversations, to Thanos Halazonetis and Ophelia Papadoulas for Myc-binding site oligonucleotides and help with the gel retardation assays, and to Donald Kufe and Ralf Hass for gifts of RNA from differentiating U-937 and HL-60 cells. We are also grateful to Don Ayer and Bob Eisenman for supplying numerous reagents and communicating their data prior to publication, and to Bob Kingston, Tod Gulick, Andrew Menzelsohn, Russ Finley, Steve Hanes, and Andrew Lassar for comments on the manuscript. A. S. Z. was supported by Hoechst AG, J. G. by a fellowship from the HFSP, and R. B. was supported by the Pew Scholar's program and an ACS faculty research award. Work was supported by Hoechst AG.

Received September 25, 1992; revised November 24, 1992

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GenBank Accession Number

The accession number for the Mx1 sequence reported in this paper is L07648.